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## 13. ABSTRACT (Maximum 200 Words)

A significant percentage of breast tumors are resistant to apoptotic stimuli. This resistance has been correlated with decreased expression of the proapoptotic protein *bax*. A major regulator of *bax* expression is the tumor suppressor p53. Unlike other well characterized p53 response elements, like the *p21-5'* element, which consist of two consensus p53 half-sites, the response element of the human *bax* promoter consists of three half-sites that cooperate in mediating p53-dependent transactivation. Within this unique response element are six GC-rich base pairs that mediate an interaction with Sp1 both *in vitro* and in cells. These bases were found to be required for p53-dependent activation, and mutations that inhibited Sp1 binding also blocked the ability of p53 to activate transcription through this element, suggesting a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human *bax* promoter. In addition, I recently identified a novel p53 response element conserved in the first intron of both the human and the murine *bax* genes. This element is required for the p53-dependent transcriptional activation of both the human and murine *bax* genes, demonstrating that *bax* is a direct and evolutionarily conserved transcriptional target of p53.

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## Differential Activation of p53 Target Genes in Breast Cancer

### Introduction:

In the past decade, there have been great advances in the field of breast cancer. These advances largely have been in the fields of education and early detection. Unfortunately, however, the treatment of breast cancer continues to be problematic in that a significant proportion of tumors are resistant to chemotherapy. Evidence suggests that this resistance is due to a defect in the normal programmed cell death or apoptotic pathway. A complete understanding of the apoptotic mechanism, therefore, is essential for designing the most therapeutic regimen for the treatment of breast cancer. The protein Bax is a key regulator of the apoptotic process, and low levels of this protein correlate with a decreased response to treatment and a decreased survival period in breast cancer patients. A major regulator of *bax* transcription is the tumor suppressor protein p53, and the work I conducted during this fellowship addressed the transcriptional regulation of the *bax* gene by p53. As such, I hope that mechanism by which Bax levels are reduced in breast tumor cells can be understood, and that this information can be used to elevate the level of activation of the *bax* gene, causing breast tumors to become sensitive to apoptotic stimuli like chemotherapy. I believe that completion of the work proposed in this fellowship will enhance our understanding of both the origins of breast cancer and how tumors respond to treatment. In addition, I believe this work will identify novel targets for therapeutic intervention in breast tumors that are otherwise resistant to current treatment.

### Body:

A significant percentage of breast tumors have been shown to be resistant to apoptotic stimuli such as chemotherapeutic drugs. This resistance has been correlated with a decreased expression of the proapoptotic protein Bax. Low Bax levels have been associated with a decreased treatment response and a shorter survival time in women with metastatic breast adenocarcinoma. In addition, overexpression of Bax in tumor cell lines sensitizes the cells to drug-induced apoptosis. A major regulator of *bax* expression is the tumor suppressor p53. p53 is a well-characterized transcription factor that can bind DNA in a sequence specific manner and activate the transcription of particular genes, including *bax*. When compared to alternate p53 targets such as the cyclin dependent kinase inhibitor *p21*, several lines of evidence suggest that the *bax* gene is differentially regulated by p53. Preliminary data presented in the fellowship application demonstrated that the breast carcinoma MCF-7 and MDA-MB-453 cell lines both exhibited a defect in the ability of wild-type p53 to activate transcription through both the intact *bax* promoter as well as the p53 response element in isolation from the promoter. This defect was specific for *bax* as p53 was capable of activating transcription of other p53-dependent target genes, such as the cyclin-dependent kinase inhibitor *p21*. The goal of the work conducted in this



## Differential Activation of p53 Target Genes in Breast Cancer

fellowship was to understand the molecular mechanism by which wild-type p53 selectively fails to activate the transcription of the pro-apoptotic *bax* gene.

Work on this project began by identifying the minimal sequence within the *bax* promoter required to mediate p53-dependent transactivation. In contrast to other well characterized p53 response elements, which consist of two copies (half-sites) of sequences that closely resemble the palendromic decamer 5'-RRRCWWGYYY-3' (where R is a purine, Y is a pyrimidine, and W is an adenine or threonine) separated from each other by 0-13 bases, the minimal element from the *bax* promoter consists of three copies of this sequence, or three half-sites. Using this minimal sequence as a probe in electrophoretic mobility shift assays (EMSAs) with nuclear extract, a factor was identified that demonstrates marked sequence specificity for this p53 response element. This factor, referred to as BBF (Bax Binding Factor) in the original fellowship application, is specific for the p53-response element of *bax* as it fails to bind other well-characterized p53-response elements, including that of the cyclin-dependent kinase inhibitor *p21* (Thornborrow and Manfredi, 1999). Five tasks were proposed in the original fellowship application to address the biological role of this binding factor in both breast tumor formation and resistance to treatment: 1) Identify additional wild-type p53 breast cancer cell lines that fail to activate transcription of the *bax* gene and fail to undergo p53-dependent apoptosis in response to DNA damage; 2) Explore a possible correlation between BBF levels and a cell line's ability to activate transcription of the *bax* gene and to undergo p53-dependent apoptosis; 3) Purify and clone BBF; 4) Determine the relevance of BBF to the apoptotic response in relevant breast cell model systems; 5) Examine the potential correlation between BBF levels, *bax* levels, and p53 status in breast tumor biopsy samples as compared to normal mammary epithelium.

Work on Task #3 successfully identified BBF as the transcription factor Sp1 (see Appendix 1). Mutational analysis demonstrated that the binding of Sp1 to the *bax* promoter is mediated by six base pairs (5'-GGGCGT-3') in the p53 half-site most proximal to the start site of transcription (Figure 4 and 8). Further, it was shown that the minimal response element capable of mediating p53-dependent transcriptional activation consists of the two distal p53 half-sites plus this adjacent six base pairs (Figure 7 and 8). This GC-rich region constitutes a "GC-box" capable both of binding members of the Sp family of transcription factors, including Sp1 *in vitro* (Figures 3 and 4), and of conferring Sp1-dependent transcriptional activation on a minimal promoter in cells (Figure 5). Mutations within this GC-box abrogated the ability of p53 to activate transcription without affecting the affinity of p53 for its binding site (Figures 6 and 8), demonstrating that these six bases are required for p53-dependent activation. In addition, a positive correlation was observed between the ability of p53 to activate transcription in cells and the ability of Sp1 to bind this response element *in vitro* (Figures 6 and 8). Mutations that inhibited Sp1 binding also blocked the ability of p53 to activate transcription through this

### Differential Activation of p53 Target Genes in Breast Cancer

element. Together, these results suggest a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human *bax* promoter (Figure 9).

The identification of a cofactor that is required for the p53-dependent transcriptional activation of the *bax* promoter suggested several possible mechanisms by which breast tumors wild-type for p53 fail to upregulate *bax* in response to damaging stimuli (Figure 9). Two independent reports, however, demonstrated that the murine *bax* promoter is unresponsive to p53 in transient transfection assays (Igata et al., 1999, Schmidt et al., 1999), suggesting a lack of evolutionary conservation with respect to the ability of p53 to transcriptionally regulate *bax* expression, and questioning the significance of the above studies done with the human *bax* promoter. Before further studies were conducted to assay the potential role of the Sp1-like cofactor in breast tumors, it became necessary to address the apparent discrepancy produced by the murine *bax* studies. To assay for additional sequence elements that influence p53-dependent transcriptional activation, extended pieces of both the human and murine *bax* genes were cloned (see Appendix 2). Sequence analysis of these two genes identified two regions conserved between human and mouse (Figure 1 and 3). The first region corresponds to the previously characterized p53 response element in the human *bax* promoter at -113 to -83 from the start site of transcription (Figure 1). The second region of conservation is found in the first intron of both genes and resembles the p53 DNA-binding consensus sequence (Figure 3). Both regions in the human and murine *bax* genes were capable of binding p53 in a sequence specific manner *in vitro* (Figures 2 and 3) and of conferring p53 responsiveness upon a minimal promoter in cells (Figures 2 and 4). Further, it was found that the p53 response element in the first intron of the human and murine *bax* genes is both required and sufficient for p53-dependent transcriptional activation in the context of a transient assay (Figures 4 and 5). Studies examining endogenous *bax* levels further supported an important role for the intronic response element (Figure 6 and 7). Additional studies suggested that the promoter response element is capable of mediating p53-dependent transcription but that in the larger context of the gene it may be inhibited by sequences contained within the 5'-untranslated region (Figure 2).

The studies characterizing the novel p53 response element in the first intron of both the human and murine *bax* genes were a digression from the original Statement of Work. These experiments, however, were essential to validate the significance of the work done with the human *bax* promoter. Prior to continuing with the original tasks, it is important to determine the relevance of both the promoter and intronic response elements to the p53-dependent upregulation of endogenous *bax* expression in response to physiological stresses like DNA damage. Once it is established that either one or both of these elements is required for p53-dependent upregulation of *bax* expression, then subsequent questions concerning the potential role of coactivating elements as well as possible mechanisms of p53 action, including roles in chromatin remodeling and/or transcriptional elongation, can be addressed.

## **Differential Activation of p53 Target Genes in Breast Cancer**

In addition to the above research accomplishments, I have continued to enhance my academic training and educational experience. While my course work was completed in 2000, I continued to participate in a weekly journal club, research seminar, and student club, each focussing on issues that are particularly relevant to the topics of tumor formation and progression. Also, I have had the opportunity to attend international scientific meetings, including the 91st Annual Meeting of the American Association for Cancer Research and the 10th International p53 Workshop, both of which have allowed me to interact with renowned scientists in the field of tumor biology, and to learn of the latest developments in the field. To be properly prepared for a successful career in the battle against breast cancer one should be familiar with both the history of research as well as the most current techniques used in the field. I believe that the work I conducted was so intimately connected to both the origins and the treatment of breast cancer that it will provide me with the broad foundation of knowledge and technical skill that will allow me to continually identify important clinical aspects of breast cancer that can benefit from further scientific research. As an M.D./Ph.D. student at the Mount Sinai School of Medicine I am receiving specialized training that will allow me to traverse both the clinical and basic science worlds. The research I conducted in the laboratory during this fellowship was an essential compliment to my clinical training that will continue to provide me with the skills to decrease the gap between benchtop discoveries and bedside cures. The experience of both designing my research project and scrutinizing my experimental data helped me to develop the analytical skills and experience necessary for a productive future battling breast cancer.

### **Key Research Accomplishments:**

- Identified minimal p53 responsive element in the human *bax* promoter.
  - Found the response element to be unique in that it consists of three p53 half-sites instead of the typical two.
- Demonstrated that p53 requires Sp1 or an Sp1-like cofactor to mediate transcriptional activation of the human *bax* promoter.
- Identified a novel p53 response element perfectly conserved in the first intron of both the human and murine *bax* genes.
  - Demonstrated that this element is both required and sufficient for the p53-dependent transcriptional activation of *bax*.

### **Reportable Outcomes:**

## Differential Activation of p53 Target Genes in Breast Cancer

### Papers

**Edward C. Thornborrow** and James J. Manfredi (2001) The Tumor Suppressor Protein p53 Requires a Cofactor to Activate Transcriptionally the Human *BAX* Promoter, *Journal of Biological Chemistry* **276**: 15598-15608.

**Edward C. Thornborrow**, Sejal Patel, Anthony E. Mastropietro, Elissa M. Schwartzfarb, and James J. Manfredi (2002) A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine *bax* genes, *Oncogene* **21**: 990-999.

### Abstracts

**Thornborrow, E. C.**, and J. J. Manfredi (2000) Identification of a High-affinity Sp1 binding site within the p53-response Element of the Human *bax* Promoter. *Tenth International p53 Workshop*.

### Degrees Obtained

Mount Sinai School of Medicine of New York University, New York, NY

Medical Scientist Training Program (Combined M.D./Ph.D.)

**2001** Ph.D. in Biomedical Science—Molecular Basis of Disease and Therapy

Advisor: Dr. James J. Manfredi

Thesis: *Transcriptional regulation of the proapoptotic bax gene by the tumor suppressor protein p53*

### Conclusions:

Low levels of Bax protein correlate with a decreased response to treatment and a decreased survival period in breast cancer patients. A major regulator of *bax* transcription is the tumor suppressor protein p53. The work conducted during this fellowship has analyzed in detail the interaction of p53 with the *bax* gene, and demonstrated that p53 requires both a cofactor to function through the *bax* promoter and a newly identified intronic response element to function in the context of the *bax* gene. Both of these observations suggest novel mechanisms to explain the defect in p53-dependent *bax* expression and apoptosis observed in many breast tumors. I believe that completion of the work proposed in this fellowship will enhance our understanding

### **Differential Activation of p53 Target Genes in Breast Cancer**

of both the origins of breast cancer and how tumors respond to treatment. In addition, I believe this work may identify novel targets for therapeutic intervention in breast tumors that are otherwise resistant to current treatment. Perhaps more significantly, this fellowship has allowed me to obtain an exceptional education that will form the basis for a continuing career in the battle against breast cancer.

#### **References:**

- Igata, E., Inoue, T., Ohtani-Fujita, N., Sowa, Y., Tsujimoto, Y. and Sakai, T. (1999) *Gene*, 238, 407-415.
- Schmidt, T., Korner, K., Karsunky, H., Korsmeyer, S., Muller, R. and Moroy, T. (1999) *Cell Death Differ*, 6, 873-882.
- Thornborrow, E. C. and Manfredi, J. J. (1999) *J Biol Chem*, 274, 33747-33756.

**Differential Activation of p53 Target Genes in Breast Cancer**

**Appendices:**

Appendix 1: "The Tumor Suppressor Protein p53 Requires a Cofactor to Activate Transcriptionally the Human *BAX* Promoter"

Appendix 2: "A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine *bax* genes"

**Personnel Receiving Pay from the Research Effort:**

Edward C. Thornborrow

## The Tumor Suppressor Protein p53 Requires a Cofactor to Activate Transcriptionally the Human BAX Promoter\*

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An important regulator of the proapoptotic BAX is the tumor suppressor protein p53. Unlike the *p21* gene, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, it previously was reported that activation of the BAX element by p53 requires additional sequences. Here, it is demonstrated that the minimal BAX response element capable of mediating p53-dependent transcriptional activation consists of two p53 half-sites plus an adjacent 6 base pairs (5'-GGGCGT-3'). This GC-rich region constitutes a "GC box" capable both of binding members of the Sp family of transcription factors, including Sp1 *in vitro*, and of conferring Sp1-dependent transcriptional activation on a minimal promoter in cells. Mutations within this GC box abrogated the ability of p53 to activate transcription without affecting the affinity of p53 for its binding site, demonstrating that these 6 bases are required for p53-dependent activation. In addition, a positive correlation was observed between the ability of p53 to activate transcription in cells and the ability of Sp1 to bind this response element *in vitro*. Mutations that inhibited Sp1 binding also blocked the ability of p53 to activate transcription through this element. Together, these results suggest a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human BAX promoter.

The BCL-2 family of proteins are key mediators of the apoptotic response. One member of this family is the proapoptotic BAX. Preceding apoptosis, cytosolic BAX translocates to the mitochondria and homodimerizes. Homodimeric BAX then is thought to cause the release of cytochrome *c* (1–3) which subsequently functions as a coactivator of Apaf-1 in the cleavage of pro-caspase-9, initiating programmed cell death (4). BAX exists in equilibrium with two of its homologs, BCL-2 and BCL-X<sub>L</sub>. Unlike BAX, these two homologs exert antiapoptotic effects by heterodimerizing with BAX in the mitochondria, blocking its ability to release cytochrome *c* (5, 6). Thus, an important determinant of the apoptotic response of a cell is the balance between the levels of BAX and BCL-2/BCL-X<sub>L</sub>. In this regard, regulation of the level of expression of BAX protein is key.

\* This work was supported by NCI Grant CA69161 from the National Institutes of Health and the Breast Cancer Program of the United States Army Medical Research and Materiel Command Grants DAMD-17-97-1-7336, DAMD-17-97-1-7337, and DAMD-17-99-1-9308. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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An important regulator of BAX gene expression is the tumor suppressor protein p53 (7, 8). The p53 protein has been implicated in several growth-related pathways, including apoptosis and cell cycle arrest (9, 10). The ability of p53 to function as a sequence-specific DNA-binding protein appears to be central to its role as a tumor suppressor (11, 12). At its amino terminus, the protein contains a potent transcriptional activation domain (13) that is linked to a central core domain that mediates sequence-specific DNA binding (14–16). Both of these domains have been shown to be important for p53-mediated growth suppression (17).

A DNA consensus sequence through which p53 binds and activates transcription has been identified. This sequence consists of two palindromic decamers of 5'-RRRCWWGYYY-3' (where R is a purine; Y is a pyrimidine; and W is an adenine or thymine) separated by 0–13 base pairs, forming four repeats of the pentamer 5'-RRRCW-3' alternating between the top and bottom strands of the DNA duplex (18, 19). Through sequences similar to this consensus, p53 has been shown to activate the transcription of many genes, including BAX, *p21*, *mdm2*, *gadd45*, *IGF-BP3*, and *cyclin G* (8, 20–26). When compared with alternate p53 targets, studies demonstrate that the BAX gene is differentially regulated by wild-type p53 in a cell type-specific manner (7, 27, 28). In the mouse, p53-dependent regulation of BAX expression following ionizing radiation is seen in the prostate, thymus, spleen, small intestine, and lung, as well as sympathetic, Purkinje, and olfactory cortical neurons. In the kidney, heart, liver, and brain, however, no p53-dependent regulation of BAX is observed (7, 27). Furthermore, the myeloid leukemia ML-1, Burkitt's lymphoma WMN and AG876, and lymphoblastoid NL2 and FWL cell lines induce BAX following ionizing radiation, whereas the fibroblast AG1522 and WI38, colorectal carcinoma RKO, and osteosarcoma U2-OS cell lines fail to do so (28). In addition, several tumor-derived p53 mutants have been identified that are capable of activating transcription through the promoter of the *p21* gene but not through the BAX promoter (29–32). This correlates with an inability of these mutants to trigger apoptosis (29, 31, 32), suggesting that a failure in the ability of p53 to transactivate the BAX gene may play an important role in tumor formation and progression. Supporting this, Yin *et al.* (33) demonstrated that BAX is an obligatory downstream effector for the p53-mediated apoptosis that attenuates choroid plexus tumor growth in the TgT121 mouse model. Thus, a complete understanding of the transcriptional regulation of the BAX promoter by p53 may yield important information relevant to our understanding of tumorigenesis.

Here is presented a detailed analysis of the p53 response element located in the promoter of the human BAX gene. The minimal BAX response element capable of mediating p53-dependent transcriptional activation is found to consist of two p53 half-sites plus an adjacent 6 base pairs (5'-GGGCGT-3') that



**Electrophoretic Mobility Shift Assay**—Production of baculovirus-infected Sf9 cell extracts and purification of recombinant human p53 protein were done as described previously (34). Purified p53 protein,

## EXPERIMENTAL PROCEDURES

**Cells**—The osteosarcoma Saos-2 cell line was maintained in a humidified tissue culture incubator at 37 °C with 5% CO<sub>2</sub>. Cells were grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. *Drosophila* SL2 cells were cultured at 25 °C in Schneider's *Drosophila* medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

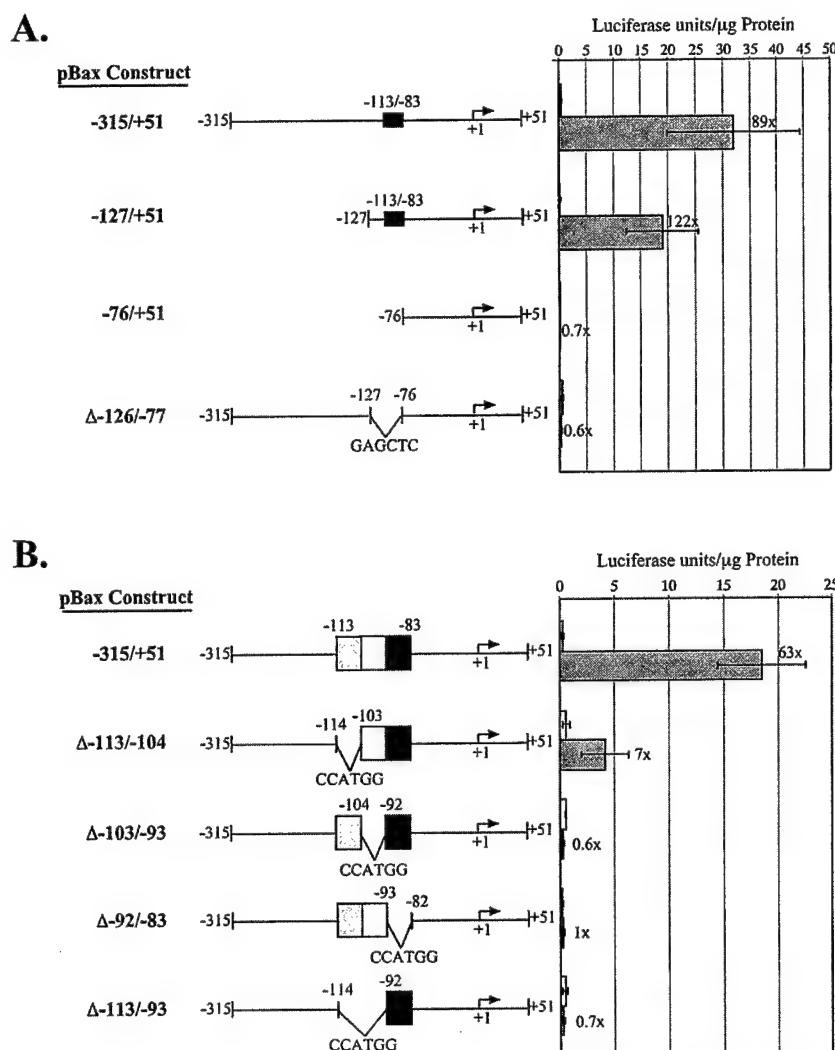
*Oligonucleotides*—For use in electrophoretic mobility shift assays and for subsequent cloning into luciferase reporter plasmids, complementary single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides with the following indicated sequences: BAX -113/-77, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGCTAGCGAATT; BAX -113/-83, AATTCGTACCTCACAAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; BAX -113/-92, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTGCTAGCGAATT; BAX -102/-83, AATTCGGTACCAGAACGCTGGGCGTGGGCGCTAGCGAATT; BAX-113/-83(sc -102/-93), AATTCGGTACCTCACAAAGTTAGCTACCTAAGGGGCGTGGGCGCTAGCGAATT; BAX(-113/-93)3, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTCACTGGTACAAAGTTAGAGACAAGCCTGCTAGCGAATT; BAX -92/-83, AATTCGGTACCGGGCGTGGGCGCTAGCGAATT; p21 5', AATTCGGTACCGAA-CATGTCCCCAATGTTGGCTAGCGAATT; BAX/p21 5' hybrid, AATTCGGTACCAGACAAGCCTCAACATGTTGGCTAGCGAATT; p21 5'/BAX hybrid, AATTCGGTACCAGAACATGTGCGGGCGTGGGCGCTAGCGAATT; Sp1 consensus, ATTCGATCGGGGCGGGGCGGAGC-BAXG-92/-91AA, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTTAAGCGTGGGCGCTAGCGAATT; BAXG-85/-84AA, AATTCGGT-ACCTCACAAAGTTAGAGACAAGCCTGGGCGTGAACGCTAGCGAAT-T; BAXG-92A, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTAG-CGCTGGGCGCTAGCGAATT; BAXG-91A, AATTCGGTACCTCACAA-AGTTAGAGACAAGCCTGAGCGTGGGCGCTAGCGAATT; BAXG-90A, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTGGACGTGG-CGCTAGCGAATT; BAXC-89A, AATTCGGTACCTCACAAAGTTAGA-GACAAGCCTGGGAGTGGGCGCTAGCGAATT; BAXG-88A, AATTC-GGTACCTCACAAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGA-ATT; BAXT-87G, AATTCGGTACCTCACAAAGTTAGAGACAAGCCT-GGGCGGGGGCGCTAGCGAATT; BAXG-86T, AATTCGGTACCTCA-CAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; BAXG-85T, AATTCGGTACCTCACAAAGTTAGAGACAAGCCTGGGCGTGT-GCGCTAGCGAATT; BAXG-84T, AATTCGGTACCTCACAAAGTTAGA-GACAAGCCTGGGCGTGGTTCGCTAGCGAATT; BAXC-83A, AATTC-GGTACCTCACAAAGTTAGAGACAAGCCTGGGCGTGGGAGCTAGCG-AATT; BAX sc -92/-83, AATTCGGTACCTCACAAAGTTAGAGACAAG-CCTTTTATGTTTAGCTAGCGAATT; BAX sc -86/-83, AATTCGGTA-CCTCACAAAGTTAGAGACAAGCCTGGGCGTTTGTAGCTAGCGAATT.

**Plasmids**—The following synthetic double-stranded oligonucleotides were digested with *Kpn*I and *Nhe*I and cloned into pGL3-E1bTATA (34), which also had been double-digested with *Kpn*I and *Nhe*I to produce pTATA vectors with corresponding names: BAX -113/-83, BAX -113/-93, BAX -102/-83, BAX -113/-83(sc -102/-93), BAXI -113/-93/3, BAX -92/-83, BAX -133/-77, p21 5', BAXGG-92/-91AA, BAXGG-85/-84AA, BAXG-92A, BAXG-91A, BAXG-90A, BAXC-89A, BAXG-88A, BAXT-87G, BAXG-86T, BAXG-85T, BAXG-84T, BAXC-83A, BAX sc -92/-83, BAX sc -86/-83. pBAX -315/+51, pBAX -127/+51, and pBAX -76/+51 were generated by PCR<sup>1</sup> amplification of the appropriate fragments from the original pBAX luciferase reporter plasmid (8). Upstream primers were engineered with the *Nhe*I restriction site. Downstream primers contained

<sup>1</sup> The abbreviations used are: PCR, polymerase chain reaction; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays.



**FIG. 1. All three potential p53 half-sites are required for the p53-dependent transcriptional activation of the human BAX promoter.** A and B, Saos-2 cells were transfected as described under "Experimental Procedures" with 1  $\mu$ g of the indicated pBAX reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53<sup>wt</sup> (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under "Experimental Procedures." The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one S.D. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53<sup>wt</sup> as compared with pCMV. A, the previously identified p53 response element is indicated by the dark gray box at -113 to -83. B, the three potential p53 half-sites are represented by the light gray (-113 to -104), white (-102 to -93), and dark gray (-92 to -83) boxes.



extract from Sf9 cells expressing recombinant human Sp1 protein, or HeLa cell nuclear extract was incubated with 3 ng of radiolabeled double-stranded oligonucleotide and antibody (Sp1 PEP-2X, p300 N-15X, and CBP 451X, Santa Cruz Biotechnology), where appropriate, in a total volume of 30  $\mu$ l of DNA binding buffer (20 mM HEPES, pH 7.5, 83 mM NaCl, 0.1 mM EDTA, 12% glycerol, 2 mM MgCl<sub>2</sub>, 2 mM spermidine, 0.7 mM DTT, and 17  $\mu$ g/ml poly(dI-dC)) for 20 min at room temperature. Samples were loaded on a native 4% acrylamide gel in 0.5 $\times$  TBE and electrophoresed at 4  $^{\circ}$ C at 225 V for 2 h. The gel was dried and exposed to Kodak XAR film using an intensifying screen at -70  $^{\circ}$ C. Phosphorimaging and densitometry data were collected with a Personal Molecular Imager FX and a GS-710 Calibrated Imaging Densitometer (Bio-Rad), and analyzed with Quantity One software (Bio-Rad).

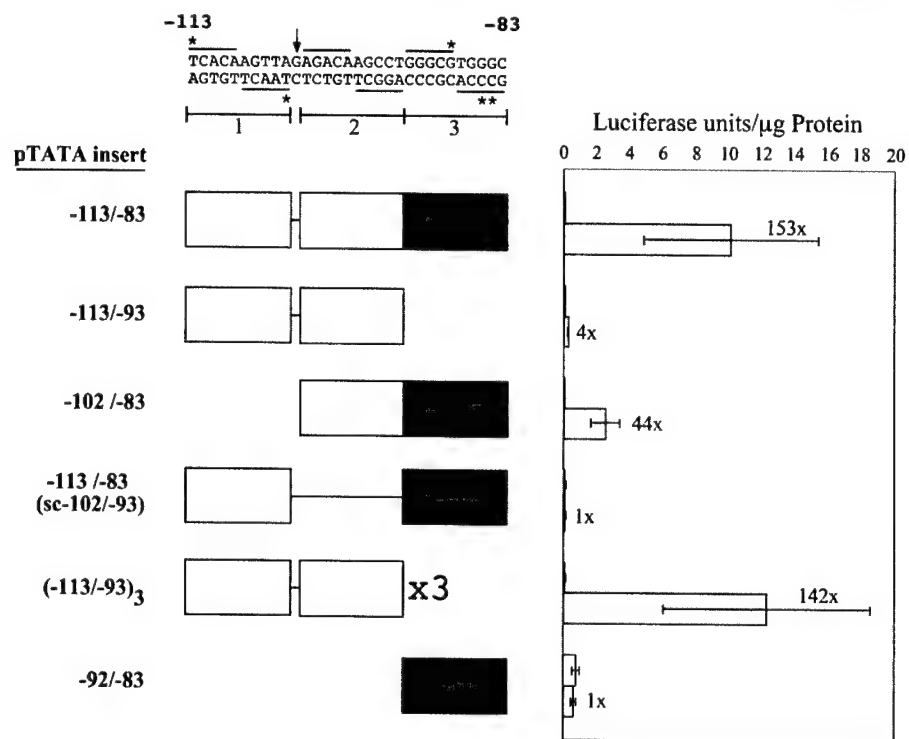
## RESULTS

**All Three Potential p53 Half-sites Are Required for the p53-dependent Transcriptional Activation of the Human BAX Promoter**—Previously it was demonstrated that in isolation the p53 response element from the human BAX promoter required sequences from three adjacent half-sites to confer p53-dependent transcriptional activation on a minimal promoter (37). To confirm the requirement of all three half-sites in the context of the BAX promoter, luciferase reporter plasmids with various deletions in the BAX promoter, both in and around the p53 response element, were cotransfected with either pCMV or a wild-type p53 expression vector into the p53-negative osteosarcoma Saos-2 cell line (Fig. 1). The previously characterized p53 response element of the BAX promoter is contained within the sequence from -113 to -83 from the start site of transcription.

There was no significant difference between the p53-dependent transactivation of either a reporter construct lacking sequences 5' to the p53 response element (pBAX -127/+51) or the full-length promoter construct (pBAX -315/+51) (Fig. 1A). Deletion of a larger fragment, including the p53 response element (pBAX -76/+51), produced a reporter construct that was unresponsive to wild-type p53 (Fig. 1A). Furthermore, targeted deletion of the promoter region containing the p53 response element (pBAX $\Delta$ -126/-77) also produced a reporter plasmid that was unresponsive to wild-type p53 (Fig. 1A). These results show that -113 to -83 is the only region, within the 366-base pair promoter fragment investigated, that affects the ability of p53 to activate transcription.

The region from -113 to -83 contains three potential p53 half-sites (represented in Fig. 1B as the light gray, white, and dark gray boxes). The role of each of these half-sites in the p53-dependent activation of the BAX promoter was examined. Removal of the first half-site from -113 to -104 (pBAX $\Delta$ -113/-104) significantly reduced the ability of p53 to activate transcription through this promoter (Fig. 1B, compare 63-fold with pBAX -315/+51 to 7-fold with pBAX $\Delta$ -113/-104), whereas removal of the second (pBAX $\Delta$ -103/-93) or the third half-site (pBAX $\Delta$ -92/-83) completely abolished the ability of p53 to activate transcriptionally the promoter (Fig. 1B). Consistent with the above results, removal of the first and second half-sites in combination (pBAX $\Delta$ -113/-93) also abolished the ability of p53 to activate transcriptionally the promoter (Fig. 1B).

**FIG. 2. The first two potential p53 half-sites constitute a *bona fide* p53 response element.** Saos-2 cells were transfected as described under "Experimental Procedures" with 1  $\mu$ g of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53<sup>wt</sup> (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under "Experimental Procedures." The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53<sup>wt</sup> as compared with pCMV. The sequence of the BAX promoter from -113 to -83 is given at the top of the figure. Potential p53 quarter-sites are indicated by the solid bars above and below the sequence. Bases that deviate from the p53 DNA-binding consensus sequence are indicated by asterisks. The three potential half-sites are indicated by the brackets labeled 1-3, respectively, and are represented graphically as the light gray, white, and dark gray boxes, respectively. The vertical arrow above the BAX sequence indicates the 1-base pair insert between the first and second half-sites.

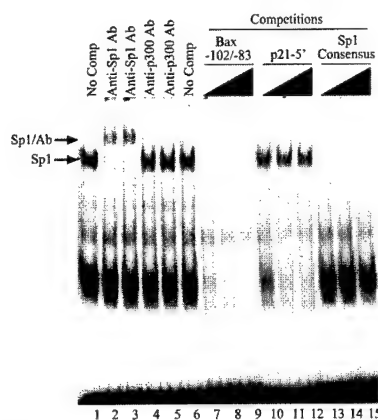


These results demonstrate that, as was observed with the isolated response element (37), p53 requires sequences from all three potential half-sites to mediate transcriptional activation of the BAX promoter.

**The First Two Potential p53 Half-sites Constitute a *Bona Fide* p53 Response Element**—Each of the three potential p53 half-sites located in the BAX promoter from -113 to -83 closely resembles the consensus sequence of 5'-RRRCWW-GYYY-3' (represented in Fig. 2 by the light gray, white, and dark gray boxes). The first, located at -113 to -104, deviates from the consensus at 2 bases (-113 and -104). The second half-site matches the consensus sequence at all 20 base pairs and is located at -102 to -93. The third half-site is located at -92 to -83 and deviates from the consensus at three bases (-84, -85, and -88) (see Fig. 2). These three half-sites can combine in different ways to produce a total of three possible p53 complete binding sites (half-sites 1 and 2, 2 and 3, and 1 and 3). Previous studies demonstrated that in electrophoretic mobility shift assays (EMSA), double-stranded oligonucleotides representing both -113 to -93 (half-sites 1 and 2) and -102 to -83 (half-sites 2 and 3) are capable of binding p53 in a sequence-specific manner with similar affinities (37). When cloned upstream of the adenovirus *E1b* minimal promoter in the pTATA luciferase reporter plasmid, however, the combination of the first and second half-sites (-113 to -93) is unable to mediate p53-dependent transcriptional activation (37). To examine further the ability of p53 to interact with this sequence in cells, the -113 to -93 sequence was multimerized (as three copies) and cloned into the pTATA luciferase reporter plasmid. This reporter plasmid was cotransfected with either pCMV or a wild-type p53 expression vector in the Saos-2 cell line (Fig. 2). These three copies of this p53-binding site were capable of mediating a significant degree of activation in response to p53 (Fig. 2, compare 4-fold with pTATA-113/-93 to 142-fold with pTATA(-113/-93)<sub>3</sub>), demonstrating that the sequence from -113 to -93 is indeed a *bona fide* p53 response element capable of both binding p53 in a sequence-specific manner *in vitro* and mediating p53-dependent transcriptional activation in cells. Confirming previous results, p53 was able to activate transcription through the second and third half-sites (-102 to

-83), but this activation was significantly reduced as compared with that mediated by all three half-sites combined (Fig. 2, compare 44-fold with pTATA-102/-83 and 153-fold with pTATA-113/-83). To test the ability of half-sites one and three to mediate p53-dependent transcriptional activation, a synthetic oligonucleotide corresponding to -113 to -83 of the BAX promoter, with -102 to -93 scrambled to remove any contribution of the second half-site, was cloned into the pTATA reporter plasmid. This construct failed to be activated by p53 (Fig. 2, pTATA-113/-83(sc -102/-93)). The third half-site in isolation (-92 to -83) also failed to mediate p53-dependent transcriptional activation (Fig. 2, pTATA-92/-83).

**Sp1 Binds with Sequence Specificity to and Activates Transcription through the p53 Response Element from the Human BAX Promoter**—We previously reported the identification of a nuclear factor, termed Binder of BAX 1 (BoB1), that interacts with sequence specificity with the same region of the human BAX promoter that is required for p53-dependent transcriptional activation (37). These previous studies demonstrated that this factor binds to sequences within the region of -102 to -83. Analysis of this region using a MatInspector search of the TRANSFAC data base (38, 39) showed that it contains a sequence that potentially could bind the transcription factor Sp1. To test this, a synthetic oligonucleotide corresponding to -102 to -83 of the BAX promoter was used as a radiolabeled probe in an EMSA with HeLa cell nuclear extract (Fig. 3). As reported previously for Saos-2 (37), HeLa cell nuclear extract contains a factor that demonstrated marked sequence specificity for the labeled BAX probe. This factor was successfully competed by increasing amounts of unlabeled probe (Fig. 3, lanes 7-9) as well as by increasing amounts of oligonucleotide corresponding to the DNA-binding consensus sequence of Sp1 (Fig. 3, lanes 13-15). This binding was specific, as an oligonucleotide corresponding to the 5' p53 response element from the human p21 promoter failed to compete for binding (Fig. 3, lanes 10-12). In addition, this factor was successfully bound by an anti-Sp1 antibody, as demonstrated by a "supershifted" complex (Fig. 3, lanes 2 and 3), whereas a control anti-p300 antibody failed to bind the factor (Fig. 3, lanes 4 and 5). Together, these data demon-

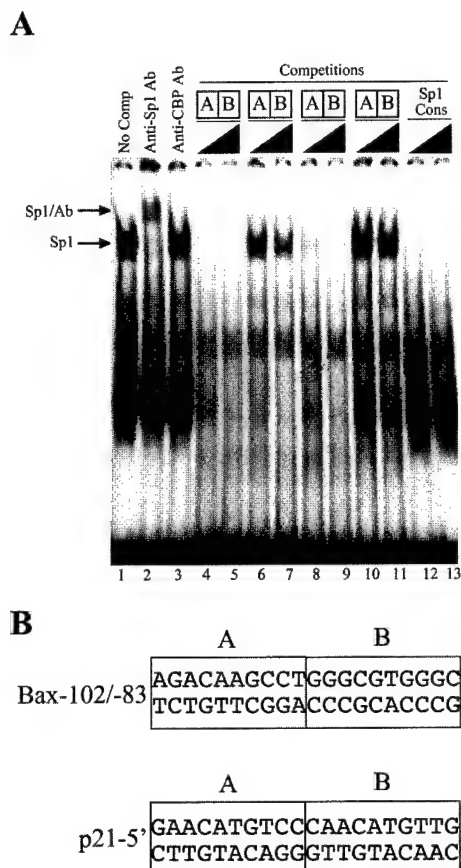


**FIG. 3. Sp1 binds with sequence specificity to the p53 response element from the human BAX promoter.** An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -102/-83 sequence from the human BAX promoter as radiolabeled probe. 8  $\mu$ g of HeLa cell nuclear extract was incubated with 3 ng of the probe alone (lanes 1 and 6), in the presence of 2 or 4  $\mu$ l of anti-Sp1 antibody (Ab) (lanes 2 and 3, respectively), 2 or 4  $\mu$ l of anti-p300 antibody (lanes 4 and 5, respectively), a 100-, 200-, or 300-fold molar excess of either the unlabeled BAX -102/-83 oligonucleotide (lanes 7-9) or p21 5' oligonucleotide (lanes 10-12), or a 10-, 20-, or 30-fold molar excess of the unlabeled Sp1 consensus oligonucleotide (lanes 13-15). The arrows indicate the positions of the Sp1-DNA complex and the supershifted complex containing antibody, Sp1, and DNA.

strate that Sp1 can bind a portion of the p53 response element from the human BAX promoter in a sequence-specific manner.

To delineate further the sequences important for Sp1 binding, oligonucleotides were synthesized that replaced portions of the BAX sequence with corresponding sequence from the p21 5' p53 response element. The sequence from -102 to -83 in the BAX promoter contains two p53 half-sites (-102 to -93 and -92 to -83), and the p21 5' element also consists of two p53 half-sites. Hybrid oligonucleotides were synthesized in which the first of the two half-sites in the BAX element was combined with the second half-site of the p21 5' element and vice versa. The oligonucleotide corresponding to -102 to -83 of the BAX promoter again was used as a radiolabeled probe with HeLa nuclear extract in an EMSA (Fig. 4). Competitions, using unlabeled probe as well as the oligonucleotides corresponding to the p21 5' element and the two hybrid elements, were conducted. Sp1 bound the radiolabeled probe (Fig. 4, lane 1) and was recognized by an anti-Sp1 antibody (Fig. 4, lane 2) but not by a control anti-CBP antibody (Fig. 4, lane 3). Both unlabeled probe and the Sp1 DNA-binding consensus site oligonucleotide effectively competed for Sp1 binding (Fig. 4, lanes 4-5 and 12-13, respectively), whereas the p21 5' element did not (Fig. 4, lanes 10-11). Consistent with the notion that Sp1 binds DNA through GC box regions, the hybrid oligonucleotide in which the first half-site is derived from the p21 sequence and the second half-site from the BAX sequence (-92 to -83, 5'-GGGCGTGGGC-3') effectively competed for Sp1 binding (Fig. 4, lanes 8-9), whereas the other hybrid oligonucleotide that replaces this GC-rich region with sequence from the p21 5' element demonstrated a significantly reduced affinity for Sp1 binding (Fig. 4, lanes 6-7). These data indicate that Sp1 binds to sequence within -92 to -83 of the BAX promoter.

To determine whether or not Sp1 can interact with this element in cells, a pTATA luciferase reporter plasmid containing -113 to -77 of the human BAX promoter was cotransfected with increasing amounts of an Sp1 expression vector into the Sp1-deficient *Drosophila* SL2 cell line (Fig. 5). Expression of Sp1 successfully activated transcription of this reporter and yet failed to activate transcription of a control plasmid containing the 5' p53 response element of the p21 promoter (Fig. 5).



**FIG. 4. The Sp1-binding site is localized to a GC-rich region of the p53 response element.** A, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -102/-83 sequence from the human BAX promoter as radiolabeled probe. 8  $\mu$ g of HeLa cell nuclear extract was incubated with 3 ng of the probe alone (lane 1), in the presence of 4  $\mu$ l of anti-Sp1 antibody (Ab) (lane 2), 4  $\mu$ l of anti-CBP antibody (lane 3), a 100- or 200-fold molar excess of either the unlabeled BAX -102/-83 oligonucleotide (lanes 4 and 5), p21 5' oligonucleotide (lanes 10 and 11), BAX/p21 5' hybrid oligonucleotide (lanes 6 and 7) or p21 5'/BAX hybrid oligonucleotide (lanes 8 and 9), or a 10- or 20-fold molar excess of the unlabeled Sp1 consensus oligonucleotide (lanes 12 and 13). The arrows indicate the positions of the Sp1-DNA and the supershifted antibody-Sp1-DNA complexes. B, the sequences of the human BAX promoter from -102 to -83 from the start site of transcription (gray boxes) and the human p21 promoter from -2281 to -2262 from the start site of transcription (white boxes; corresponding to the p21 5' oligonucleotide) are shown. Each sequence is divided into two with the first half indicated as A and the second half indicated as B. Oligonucleotides in A are represented graphically according to this color and letter scheme. For example, the BAX/p21 5' hybrid oligonucleotide that corresponds to the first half of the BAX sequence followed by the second half of the p21 sequence is indicated by a gray box labeled A followed by a white box labeled B.

Consistent with the *in vitro* EMSA results, this confirms that Sp1 is capable of activating transcription through the p53 response element of the human BAX promoter.

**The Ability of Sp1 to Bind the p53 Response Element of the BAX Promoter in Vitro Correlates with the Ability of p53 to Activate Transcription through This Element in Cells**—To explore the significance of the Sp1-binding site to the ability of p53 to activate transcription through the BAX promoter, nucleotide substitutions were identified that differentially affected the ability of p53 to activate transcription through its response element in the BAX promoter (-113 to -83). Two mutated forms of the p53 response element from the BAX promoter, in which the indicated guanine bases were replaced with adenines (Fig. 6A, GG-92/-91AA and GG-85/-84AA), were cloned into the pTATA luciferase reporter plasmid. In cotransfection assays with a wild-type p53 expression vector in

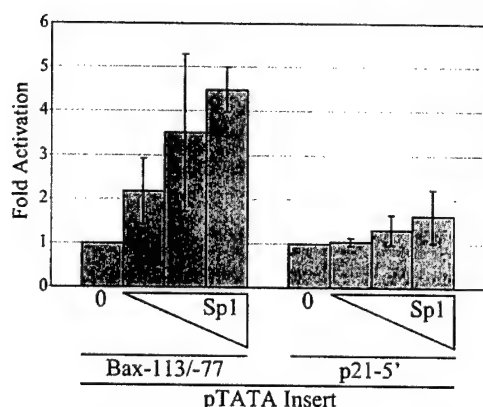


Fig. 5. **Sp1 can activate transcription through the p53 response element of the human BAX promoter.** *Drosophila* SL2 cells were transfected as described under "Experimental Procedures" with 2  $\mu$ g of the indicated pTATA reporter constructs in the presence of 0, 300, 600, or 900 ng of pPacSp1. Appropriate amounts of the vector pPacU were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.9  $\mu$ g/sample. 48 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under "Experimental Procedures." The indicated values are the average of four independent experiments expressed as the fold activation for each reporter plasmid with pPacSp1 as compared with pPacU. Error bars correspond to 1 S.D.

the Saos-2 cell line, substitution of bases -92 and -91 completely abolished the ability of p53 to activate transcription through this element (Fig. 6A, compare -113/-83 to GG-92/-91AA), whereas substitution of bases -85 and -84 did not (Fig. 6A). As observed in Figs. 1 and 2, removal of the third potential half-site (-92 to -83) inhibited the ability of p53 to mediate transcriptional activation through this element (Fig. 6A, compare -113/-83 and -113/-93), demonstrating the requirement for this Sp1-binding sequence in the p53-dependent transcriptional activation of this element.

Both of these mutant sequences were assayed for their ability to bind purified p53 in an EMSA. An oligonucleotide corresponding to -113 to -77 of the BAX promoter was used as a radiolabeled probe with purified p53 in an EMSA (Fig. 6B). Competitions were performed with increasing amounts of an oligonucleotide corresponding to -113 to -83 of the BAX promoter and the two mutant oligonucleotides. When compared with the wild-type oligonucleotide, both mutant oligonucleotides displayed a slightly decreased affinity for p53 (Fig. 6B, compare lanes 2-4 with lanes 5-7 and 8-10; Fig. 6C). Compared with one another, however, both mutant oligonucleotides demonstrated a comparable affinity for p53 (Fig. 6, A and B), suggesting that the differences in p53-dependent transcriptional activation observed in Fig. 6A are not due to differences in the affinity of p53 for the two sequences. In contrast, the abilities of the two mutant sequences to bind Sp1 differed (Fig. 6, D and E). An oligonucleotide corresponding to -113 to -77 of the BAX promoter was used as radiolabeled probe with extract from Sf9 cells expressing recombinant human Sp1 protein in an EMSA (Fig. 6D). Sp1 bound the probe and was recognized by an anti-Sp1 antibody (Fig. 6D, lanes 1-2). Sp1 binding was successfully competed by unlabeled BAX -113/-83 oligonucleotide as well as by the GG-85/-84AA mutated oligonucleotide (Fig. 6D, lanes 3-4 and 9-11, respectively; Fig. 6E). The GG-92/-91AA mutant, however, demonstrated a significant decrease in affinity for Sp1 (Fig. 6D, compare lanes 3-5 and 9-11 to lanes 6-8; Fig. 6E).

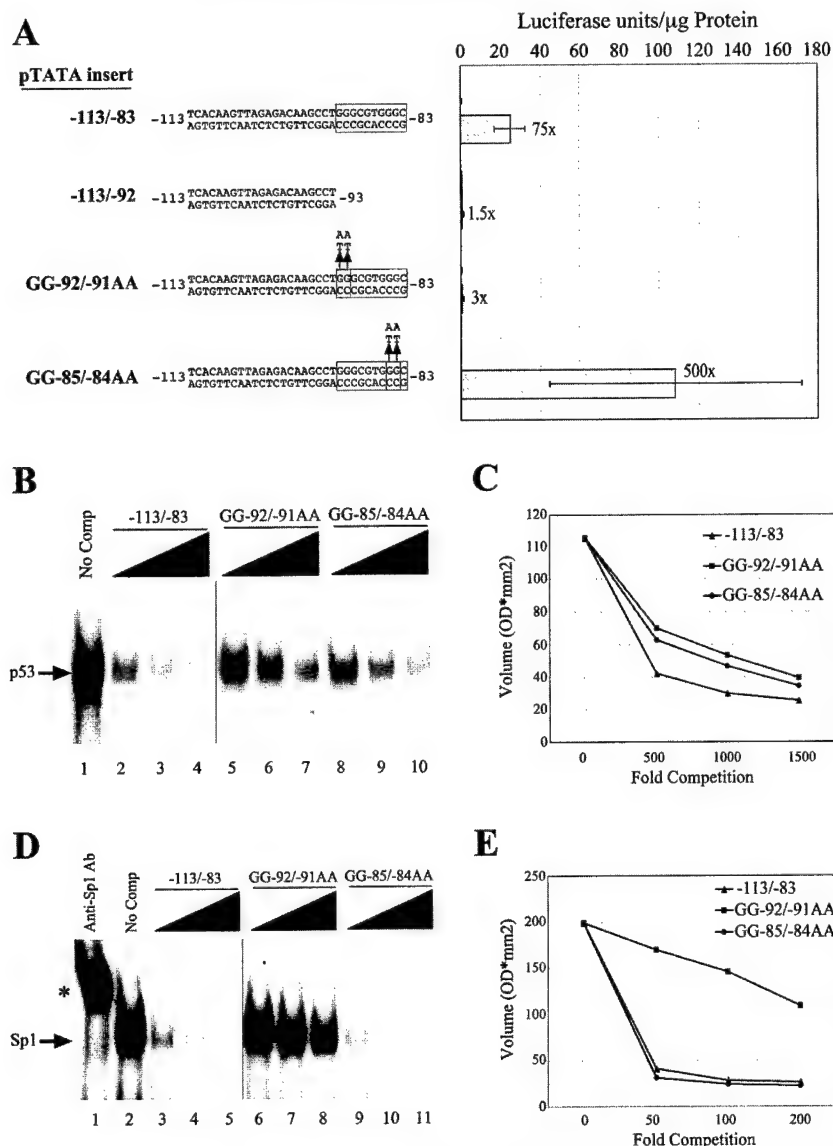
The results with the GG-85/-84AA mutant presented in Fig. 6 suggest that not all of the bases contained within the third potential half-site of the p53 response element are required for p53-dependent transcriptional activation. To iden-

tify the minimal sequence elements required to mediate p53-dependent transactivation, a series of oligonucleotides was synthesized in which each of the 10 bases of the third potential half-site (-92 to -83) was individually replaced. These mutant oligonucleotides then were cloned into the pTATA luciferase reporter plasmid and tested for their responsiveness to p53 in a cotransfection assay in the Saos-2 cell line (Fig. 7). Consistent with the results in Fig. 6A, substitution of the bases at either -85 or -84 did not inhibit the ability of p53 to activate transcription through this element (Fig. 7, G-85T and G-84T). Furthermore, substitution of -86 and -83 also failed to affect significantly the ability of p53 to activate transcription (Fig. 7, compare -113/-83 to G-86T and C-83A). Substitution of the base at -87, however, significantly reduced the ability of p53 to activate transcription through this element (Fig. 7, compare -113/-83 to T-87G). Together, these results suggest that the minimal response element consists of sequence from -113 to -87, with -86 to -83 being dispensable for p53-dependent transactivation.

To confirm that the bases from -86 to -83 are not required for p53-dependent transcriptional activation, two additional mutant oligonucleotides were synthesized. The first mutant was generated by replacing all 10 nucleotides from -92 to -83 (Fig. 8A, sc-92/-83). The 4 bases from -86 to -83 were substituted as indicated to generate the second mutant oligonucleotide (Fig. 8A, sc-86/-83). Each oligonucleotide was cloned into the pTATA vector and tested for its responsiveness to p53 in a cotransfection assay (Fig. 8A). As observed with the reporter plasmid in which the sequence from -92 to -83 is removed entirely (pTATA-113/-93), the first mutant, in which all 10 bases of the third potential half-site (-92 to -83) are replaced, showed little to no response to p53 (Fig. 8A, compare pTATA-113/-93 to pTATA-113/-83 and pTATA-sc-92/-83). In contrast, the second mutant, in which only the last 4 bases of the element (-86 to -83) are replaced, was efficiently activated by p53 (Fig. 8A, compare 312-fold with pTATA-113/-83 to 323-fold with pTATA-sc-86/-83). This result demonstrates that the minimal p53 response element in the BAX promoter consists of sequence from -113 to -87. In an EMSA both mutants displayed a decreased affinity for p53 as compared with the wild-type sequence (Fig. 8B, compare lanes 2-4 to lanes 8-10 and 11-13; Fig. 8C). When compared with each other, there was no significant difference in the affinity of p53 for the two mutant sequences (Fig. 8B, compare lanes 8-10 to 11-13; Fig. 8C). This suggests that the differences in transcriptional activation observed in Fig. 8A cannot be explained by differences in p53 affinities. Furthermore, the oligonucleotide corresponding to -113 to -93 displayed a similar affinity for p53 as the two mutant oligonucleotides (Fig. 8B, compare lanes 5-7 to lanes 8-10 and 11-13; Fig. 8C) consistent with the idea that, in the case of the two mutants, p53 is interacting with the first and the second half-sites only. The sc-86/-83 mutant oligonucleotide efficiently competed for Sp1 binding in an EMSA (Fig. 8D, compare lanes 2-4 to lanes 8-10; Fig. 8E), whereas the ability of the sc-92/-83 mutant to bind Sp1 was significantly reduced compared with the wild-type sequence (Fig. 8D, compare lanes 2-4 to lanes 5-7; Fig. 8E), further strengthening the correlation between Sp1 binding *in vitro* and p53 activation in cells.

#### DISCUSSION

The data presented in this report demonstrate that the minimum p53 response element in the BAX promoter consists of the sequence from -113 to -87 from the start site of transcription. This sequence contains a p53-binding site (-113 to -93) that can function as a *bona fide* response element as demonstrated by its ability when multimerized to confer p53-depend-



**FIG. 6. A mutant element that fails to bind Sp1 *in vitro* also fails to confer p53-dependent transcriptional activation in cells.** **A**, Saos-2 cells were transfected as described under "Experimental Procedures" with 1  $\mu$ g of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53<sup>wt</sup> (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under "Experimental Procedures." The indicated values are the average of five independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in gray with the corresponding mutations indicated above. **B**, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-77 sequence from the human *BAX* promoter as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled BAX -113/-83 oligonucleotide (lanes 2-4), the GG-92/-91AA oligonucleotide (lanes 5-7), or the GG-85/-84AA oligonucleotide (lanes 8-10). The arrow indicates the position of the p53-DNA complex. The vertical bar between lanes 4 and 5 represents the removal of irrelevant lanes from the gel. **C**, bands were quantitated by densitometry. **D**, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-77 sequence from the human *BAX* promoter as radiolabeled probe. Extract from Sf9 cells expressing human recombinant Sp1 protein was incubated with 3 ng of the probe alone (lane 2), in the presence of anti-Sp1 antibody (lane 1), or in the presence of a 50-, 100-, or 200-fold molar excess of either the unlabeled BAX -113/-83 oligonucleotide (lanes 3-5), the GG-92/-91AA oligonucleotide (lanes 6-8), or the GG-85/-84AA oligonucleotide (lanes 9-11). The arrow indicates the position of the Sp1-DNA complex, and the asterisk indicates the position of the supershifted anti-Sp1-DNA complex. The vertical bar between lanes 5 and 6 represents the removal of irrelevant lanes from the gel. **E**, bands were quantitated by densitometry.

ent transcriptional activation on a minimal promoter (Fig. 2). Immediately adjacent to this p53-binding site are 6 base pairs that are GC-rich in nature (-92 to -87: 5'-GGGCGT-3'). These 6 bases are required for p53-dependent transcriptional activation as deletion or mutation of this region in the context of either the promoter or the isolated response element completely abrogates the ability of p53 to activate transcription through this sequence (Figs. 1B, 2, 6A, and 8A). The addition of these bases to the -113/-93 sequence appears to have little effect on the affinity of p53 for this sequence (Fig. 8, B and C), consistent with a model in which these 6 bases function to

recruit a co-activator as opposed to simply enhancing p53 binding. Furthermore, these 6 base pairs mediate sequence-specific binding to the Sp1 transcription factor (Figs. 3, 4, 6D, and 8D), and a positive correlation is seen between the ability of Sp1 to bind this element *in vitro* and the ability of p53 to mediate transcriptional activation through its response element in cells (Figs. 6 and 8). In addition, the results with electrophoretic mobility shift assays with the GG-92/-91AA mutant oligonucleotide (Fig. 6B) are not consistent with the published p53 DNA-binding consensus sequence of (RRRCWWGYYY)<sub>2</sub> (18, 19). This consensus allows for a purine in the first three posi-



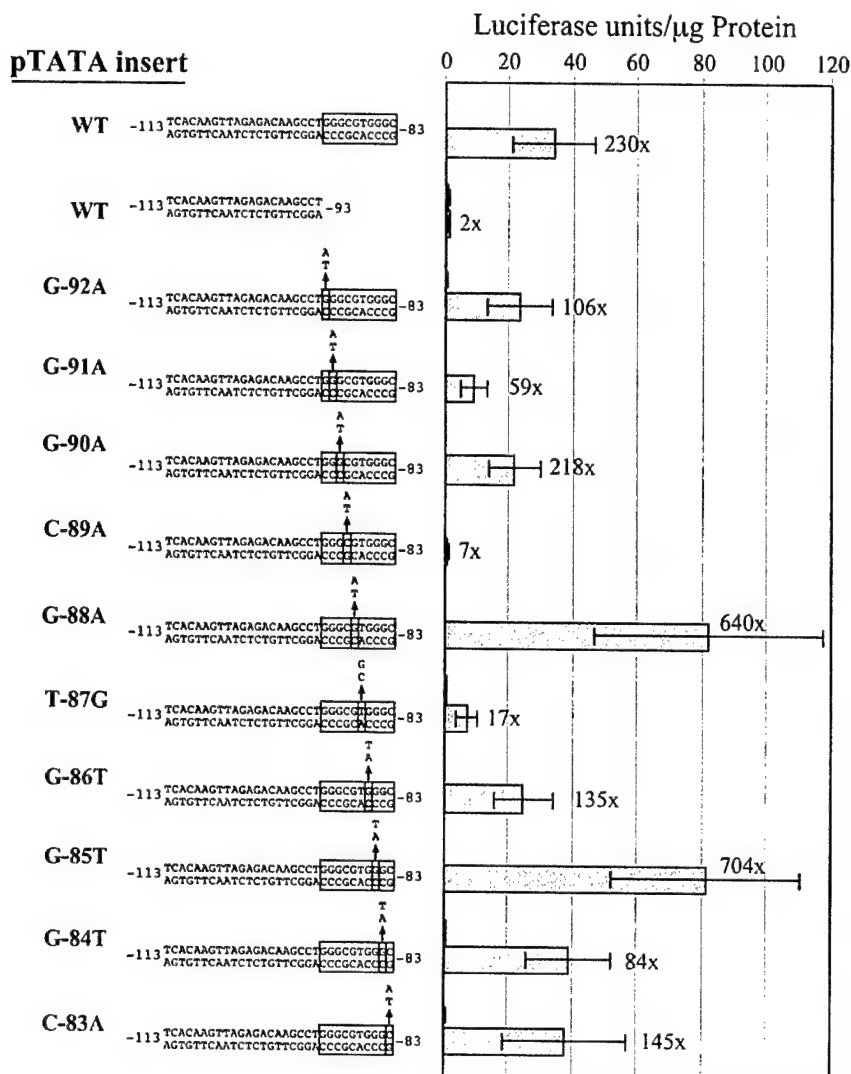


FIG. 7. Mutational analysis shows that the BAX promoter sequence from -86 to -83 is not required for p53-dependent transcriptional activation. Saos-2 cells were transfected as described under "Experimental Procedures" with 1  $\mu$ g of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53<sup>wt</sup> (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under "Experimental Procedures." The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in gray with the corresponding mutations indicated above.

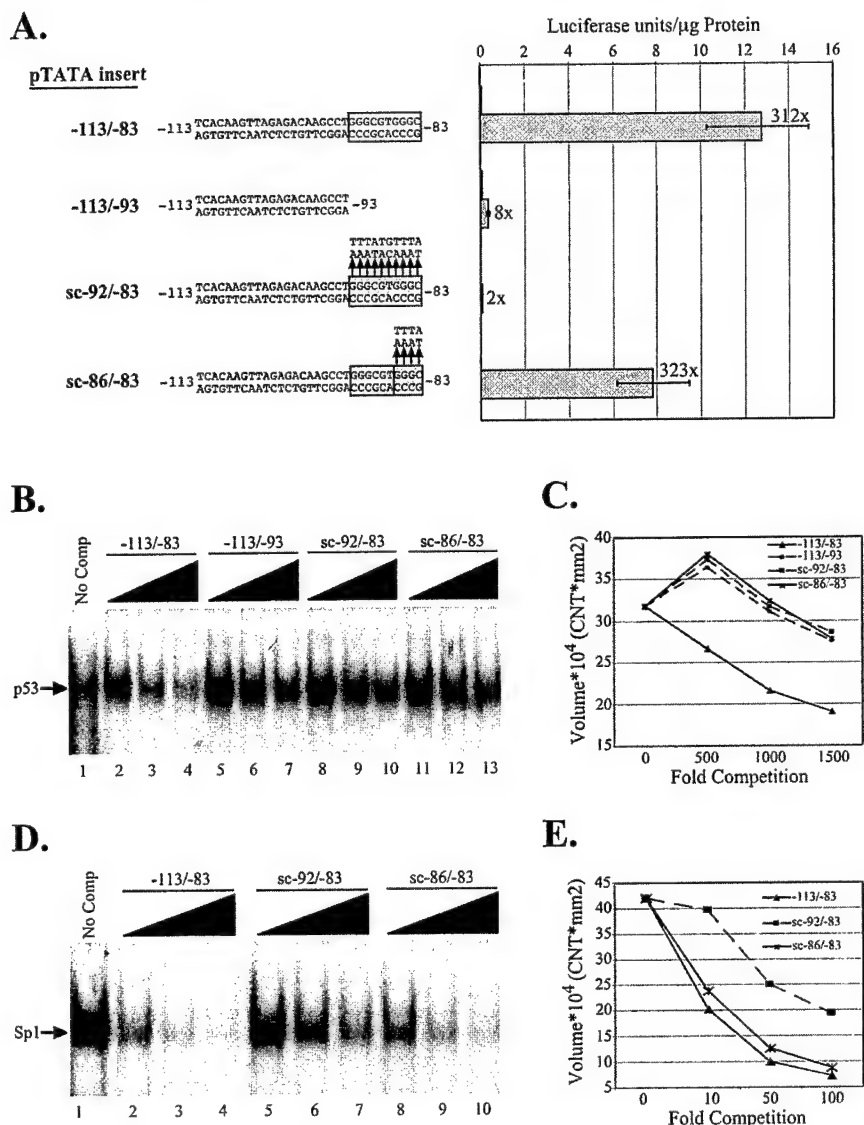
tions of each half-site. The GG-92/-91AA mutant contains a conservative substitution of purines (adenines) for purines (guanines) and, as such, does not represent a substantive change in terms of the p53 DNA-binding consensus sequence. This substitution, however, did produce a significant decrease in the ability of p53 to bind to this oligonucleotide *in vitro* (Fig. 6B), suggesting that, in these limited circumstances, the p53 DNA-binding sequence involves greater specificity than implied by the consensus.

Previous studies have suggested a connection between p53 and Sp1. The two proteins physically interact under certain circumstances (40–42), and, transcriptionally, p53 and Sp1 have been shown to function in a cooperative manner in some settings and an antagonistic manner in others (41, 43, 44). In addition to p53, Sp1 has been found to synergize with other transcription factors, including YY1 and SREBP (45–47). Studies with the Sp family of transcription factors, however, are complicated by the fact that there are at least 16 mammalian members of this family. Due to marked conservation in the DNA-binding domain, many of these family members have similar if not identical *in vitro* DNA binding characteristics (48, 49). Originally, this led to the misclassification of many GC boxes solely as Sp1-binding sites because of the ubiquitous nature of Sp1 and the fact that it was the first family member cloned. Given this, the possibility exists that the true *in vivo* cofactor required for the p53-dependent transactivation of the BAX promoter is an Sp1-related family member that is ob-

scured in *in vitro* assays by the sheer abundance of Sp1 in nuclear extracts from tissue culture cells. Consistent with this, antibodies used in a supershift EMSA identified other Sp family members as minor components of the Sp1-DNA complex.<sup>2</sup> Furthermore, cotransfection assays in the Sp1-deficient *Drosophila* SL2 cell line failed to demonstrate cooperation between Sp1 and p53 in transcriptionally activating the p53 response element of the BAX promoter.<sup>2</sup> The *Drosophila* assays, however, are difficult to interpret as the ability of p53 alone to activate transcription through a control plasmid was significantly impaired in the SL2 cell line. Complicating interpretation of the results in the *Drosophila* system is the recent identification of a *Drosophila* p53 homolog (50, 51) that may affect the ability of transiently expressed human p53 to function properly in this system.

Regardless of whether the cofactor required for the p53-dependent transactivation of the BAX promoter is Sp1 or a related family member, the requirement of this cooperating protein suggests a model for the observed cell type- and tumor type-specific regulation of the BAX gene by wild-type p53 (Fig. 9). In this model, cells that are permissive to p53-dependent up-regulation of the BAX gene express both p53 and the cofactor, and these proteins function together to activate transcriptionally the gene. In those cells that fail to show p53-dependent

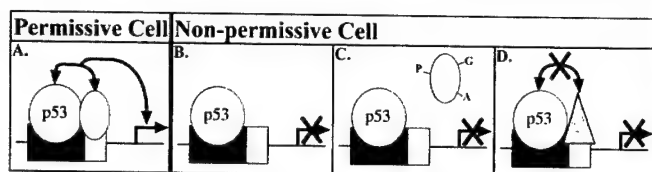
<sup>2</sup> E. C. Thornborrow and J. J. Manfredi, unpublished data.



**FIG. 8. The minimal element from the BAX promoter that confers p53-dependent transcriptional activation consists of a single p53-binding site and an adjacent Sp1-binding site.** A, Saos-2 cells were transfected as described under "Experimental Procedures" with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53<sup>wt</sup> (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under "Experimental Procedures." The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53<sup>wt</sup> as compared with pCMV. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in gray with the corresponding mutations indicated above. B, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-83 sequence from the human BAX promoter as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled BAX -113/-83 oligonucleotide (lanes 2-4), the BAX -113/-93 oligonucleotide, the BAX sc -92/-83 oligonucleotide (lanes 5-7), or the BAX sc -86/-83 oligonucleotide (lanes 8-10). The arrow indicates the position of the p53-DNA complex. C, bands were quantitated by phosphorimaging. D, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-83 sequence from the human BAX promoter as radiolabeled probe. Extract from Sf9 cells expressing human recombinant Sp1 protein was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 10-, 50-, or 100-fold molar excess of either the unlabeled BAX -113/-83 oligonucleotide (lanes 2-4), the BAX sc -92/-83 oligonucleotide (lanes 5-7), or the BAX sc -86/-83 oligonucleotide (lanes 8-10). The arrow indicates the position of the Sp1-DNA complex. E, bands were quantitated by phosphorimaging.

*BAX* expression, one can propose three possible mechanisms to explain the apparent failure of wild-type p53 to activate the *BAX* gene (Fig. 9). First, the required cofactor may be absent, either due to mutation or due to cell type-specific limitations on its expression. Second, this factor may be inactivated by post-translational modification. Finally, another factor that cannot cooperate with p53 may compete with the cofactor for binding to its site in the *BAX* promoter. Data with the Sp family of transcription factors support each of these possibilities. Although several of the Sp family members, like Sp1, are ubiquitously expressed, other members of the family display high degrees of tissue specificity (48, 49). Even the ubiquitously

expressed family members fluctuate in levels under particular cellular conditions (52-55). Sp1 mRNA, for example, varies up to 100-fold depending on the cell type and developmental stage of the mouse (56). Consistent with a model of post-translational modification, certain Sp family members, including Sp1 and EKLF, are phosphorylated, glycosylated, and acetylated (57-59). Finally, given the high level of conservation in the DNA-binding domain of the Sp family of transcription factors, it is not surprising that DNA binding competition can be observed between various members of this family. In certain cases, including Sp1/Sp3, BTEB1/AP-2rep, and BKLF/EKLF, this competition has ramifications on gene expression (60-62). In each



**FIG. 9. Model for the cell type-specific regulation of the *BAX* promoter by the tumor suppressor protein p53.** A, in cells that are permissive to p53-dependent transcriptional activation of the *BAX* gene, p53 and the required cofactor cooperate to mediate activation. In cells that do not support the p53-*BAX* pathway, three possible mechanisms may explain the apparent failure of wild-type p53 to activate the *BAX* gene. B, the cofactor may be absent due to mutation or to cell type-specific limitations on its expression. C, the cofactor may be inactivated by post-translational modification as follows: P, phosphorylation; G, glycosylation; or A, acetylation. D, another factor that cannot cooperate with p53 may compete with the required cofactor for binding to its site in the *BAX* promoter. The p53-binding site (–113 to –93) is represented by the black box. The Sp1-binding site (–93 to –87) is represented by the white box. p53, the required cofactor, and the inhibitory factor are represented by the gray circle, the dotted oval, and the cross-hatched triangle, respectively.

case, transcriptional activation by one family member is repressed by the other member by competing for the same DNA-binding site. The data in this report, in combination with the previous studies of the Sp family of transcription factors, support a model in which the regulation of a required cofactor controls cell type-specific p53-dependent expression of the *BAX* gene.

The ability of the proapoptotic *BAX* to function as a tumor suppressor protein has been substantiated by several studies. In certain mouse models, *BAX* has been shown to be an important mediator of p53-dependent apoptosis and a suppressor of oncogenic transformation, with loss of *BAX* leading to accelerated rates of tumor growth, increased tumor numbers, larger tumor mass, and decreased survival rates (63, 64). A significant correlation between decreased *BAX* expression and both a corresponding resistance to apoptotic stimuli, as well as a shorter survival period also have been observed in a number of human tumor types, including breast, ovarian, pancreatic, colorectal, and non-Hodgkin's lymphoma (65–69). In addition, in colon and gastric cancers of the microsatellite mutator phenotype mutational inactivation of the *BAX* gene has been shown to confer a strong survival advantage during tumor clonal evolution (70). Complimenting these data are observations showing that overexpression of the *BAX* protein in certain tumor cell lines both sensitizes these cells to chemotherapy- and radiation-induced apoptosis and reduces their ability to form tumors in SCID mice (71–73). Together, these results strongly support a tumor suppressor role for the *BAX* protein.

An important regulator of the *BAX* gene is the tumor suppressor protein p53. Several reports have demonstrated the significance of the p53-*BAX* pathway in tumor suppression. Both the identification of tumor-derived p53 mutants that selectively fail to activate transcription through the *BAX* promoter and subsequently fail to induce apoptosis (29–32) as well as the TgT121 transgenic studies that demonstrate that *BAX* is an obligatory downstream effector of p53 in the suppression of choroid plexus tumor growth (33) suggest that the ability of p53 to activate transcription through the *BAX* promoter is important to the tumor suppressor function of p53. Furthermore, the resistance of certain tumor cell lines to radiation therapy is associated with a failure of wild-type p53 to induce *BAX* expression (28, 74), and certain human tumors have been identified that are genetically wild-type for both p53 and *BAX* and yet fail to express significant levels of *BAX* protein (75). Thus, a complete understanding of the transcriptional regulation of the *BAX* gene by the tumor suppressor p53 may provide important information concerning both the molecular origins of

cancer as well as the development of tumor resistance to certain cancer treatments.

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# A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine *bax* genes

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Both the human and the mouse *bax* promoters contain p53 binding sites which are sufficient to confer p53-dependent transcriptional activation in a heterologous setting. Nevertheless in the context of the *bax* promoter, these sites do not mediate a p53-dependent response, suggesting that *bax* may not be a direct transcriptional target of p53. Here, data are presented identifying a conserved p53 response element in the first intron of both the human and the murine *bax* genes. This element both in isolation and in the context of the first intron conferred p53-dependent transcriptional activation upon a minimal promoter. Electrophoretic mobility shift assays demonstrated that this sequence also is capable of mediating sequence specific binding to p53. p53 effectively activated transcription through both human and murine *bax* gene reporter constructs, whereas deletion of the intronic response element abrogated the p53-responsiveness of both reporters. Interestingly, tumor-derived mutants of p53 which are defective in inducing an apoptotic response retain the ability to activate transcription via the *bax* intronic p53 site. Since these mutants are transcriptionally inactive on the p53 site in the *bax* promoter, the ability of these mutants to up-regulating endogenous *bax* mRNA levels supports a role for the intronic element in p53-dependent up-regulation of *bax* expression. Taken together, these results show the requirement for a novel intronic element in the p53-dependent transcriptional activation of *bax*, and demonstrate that *bax* is indeed a direct and evolutionarily conserved transcriptional target of p53

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**Keywords:** *bax*; p53; transcription; DNA-binding

## Introduction

Bax is an important mediator of apoptosis. In the mitochondria, homodimeric Bax is capable of releasing cytochrome *c* to the cytoplasm (Jurgensmeier *et al.*, 1998; Manon *et al.*, 1997; Pastorino *et al.*, 1998). Once

in the cytoplasm, cytochrome *c* functions as a cofactor with Apaf-1 to promote the cleavage of pro-caspase-9, initiating apoptosis (Skulachev, 1998). Two homologues, Bcl-2 and Bcl-x<sub>L</sub>, antagonize Bax function by heterodimerizing with it in the mitochondria, blocking the release of cytochrome *c* (Kim *et al.*, 1997; Oltvai *et al.*, 1993). An important determinant of the apoptotic response is the balance between the levels of Bax and Bcl-2/Bcl-x<sub>L</sub>. Regulation of Bax protein expression, therefore, is a key factor in this process.

The tumor suppressor protein p53 has been shown to regulate *bax* expression (Miyashita *et al.*, 1994; Miyashita and Reed, 1995). At its amino-terminus, the p53 protein contains a potent transcriptional activation domain (Fields and Jang, 1990) which is linked to a central core domain that mediates sequence-specific DNA binding (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). Both of these domains are important for p53-mediated growth suppression (Pietenpol *et al.*, 1994). A DNA consensus sequence through which p53 binds and activates transcription has been identified. This sequence consists of two palindromic decamers of 5'-RRRCWWGYYY-3' (where R is a purine, Y is a pyrimidine, and W is an adenine or thymine) separated by 0–13 bp (el-Deiry *et al.*, 1992; Funk *et al.*, 1992).

Miyashita and Reed (1995) identified the human *bax* gene as a transcriptional target of p53 (Miyashita *et al.*, 1994). Supporting this notion, a p53 response element in the promoter of the human *bax* gene has been demonstrated at –113 to –83 from the start site of transcription (Miyashita and Reed, 1995; Thornborrow and Manfredi, 1999). The recent cloning of the murine *bax* promoter, however, has questioned the significance of this response element. Two independent reports have demonstrated that the murine *bax* promoter is unresponsive to p53 (Igata *et al.*, 1999; Schmidt *et al.*, 1999), suggesting a lack of evolutionary conservation with respect to the ability of p53 to transcriptionally regulate *bax* expression.

To assay for additional sequence elements that influence p53-dependent transcriptional activation, extended pieces of both the human and murine *bax* genes were cloned. Data presented here is demonstrating that both the human and murine *bax* genes are direct transcriptional targets of the tumor suppressor protein p53. Sequence analysis of these two genes identified two regions conserved between human and

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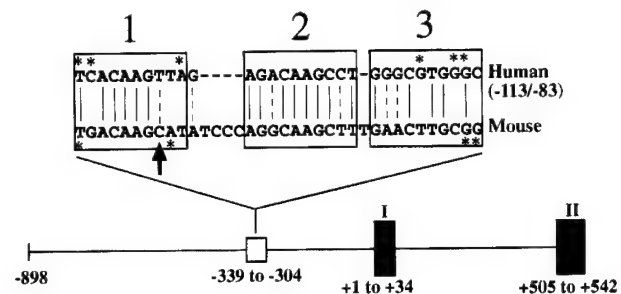
mouse. The first region corresponds to the previously characterized p53 response element in the human *bax* promoter at -113 to -83 from the start site of transcription. The second region of conservation is found in the first intron of both genes and resembles the p53 DNA-binding consensus sequence. Both regions in the human and murine *bax* genes are capable of binding p53 in a sequence specific manner *in vivo* and of conferring p53 responsiveness upon a minimal promoter in cells. Further, it is demonstrated that the p53 response element in the first intron of the human and murine *bax* genes is both required and sufficient for p53-dependent transcriptional activation.

## Results

### *The p53 response element of the human bax promoter is partially conserved in the murine promoter*

Two reports have demonstrated that the murine *bax* promoter is unresponsive to p53 in transient transfection assays (Igata et al., 1999; Schmidt et al., 1999). The p53-dependent regulation of Bax expression, however, has been observed in numerous tissues and cell types of the mouse (Miyashita et al., 1994; Bouvard et al., 2000). To explore this further, a 1440 bp fragment (-898 to +542 from the start site of translation) of the murine *bax* gene was PCR cloned (Accession #AF339055) and analysed for sequences that contribute to p53-dependent regulation. A p53 response element was previously identified in the human *bax* promoter at -113/-83 from the start site of transcription (Miyashita and Reed, 1995; Thornborrow and Manfredi, 1999). This sequence was found to be partially conserved in the murine promoter at -339/-304 from the start site of translation (Figure 1). Of the 31 bp that contribute to the p53 response element in the promoter of the human *bax* gene (Figure 1, Human(-113/-83) sequence), 19 bases are conserved in the mouse promoter (Figure 1, solid lines) and an additional six bases represent conservative substitutions of purine for purine or pyrimidine for pyrimidine (Figure 1, dashed lines). As with the human sequence, if one applies the p53 DNA-binding consensus sequence of 5'-RRRCWWGYYY-3' to this region, three potential p53 half-sites are identified (Figure 3, white boxes labeled 1-3). Of note is the Cytosine (C) at position -332 (Figure 1, arrow). This base is consistent with the murine *bax* promoter sequence published by Schmidt et al. (1999), but is not found in the sequence published by Igata et al. (1999) nor is it found in their accompanying GenBank submission (Accession #AB029557). To confirm the presence of this base, this region was PCR cloned from mouse genomic DNA on five independent occasions. In each case the C was present at position -332.

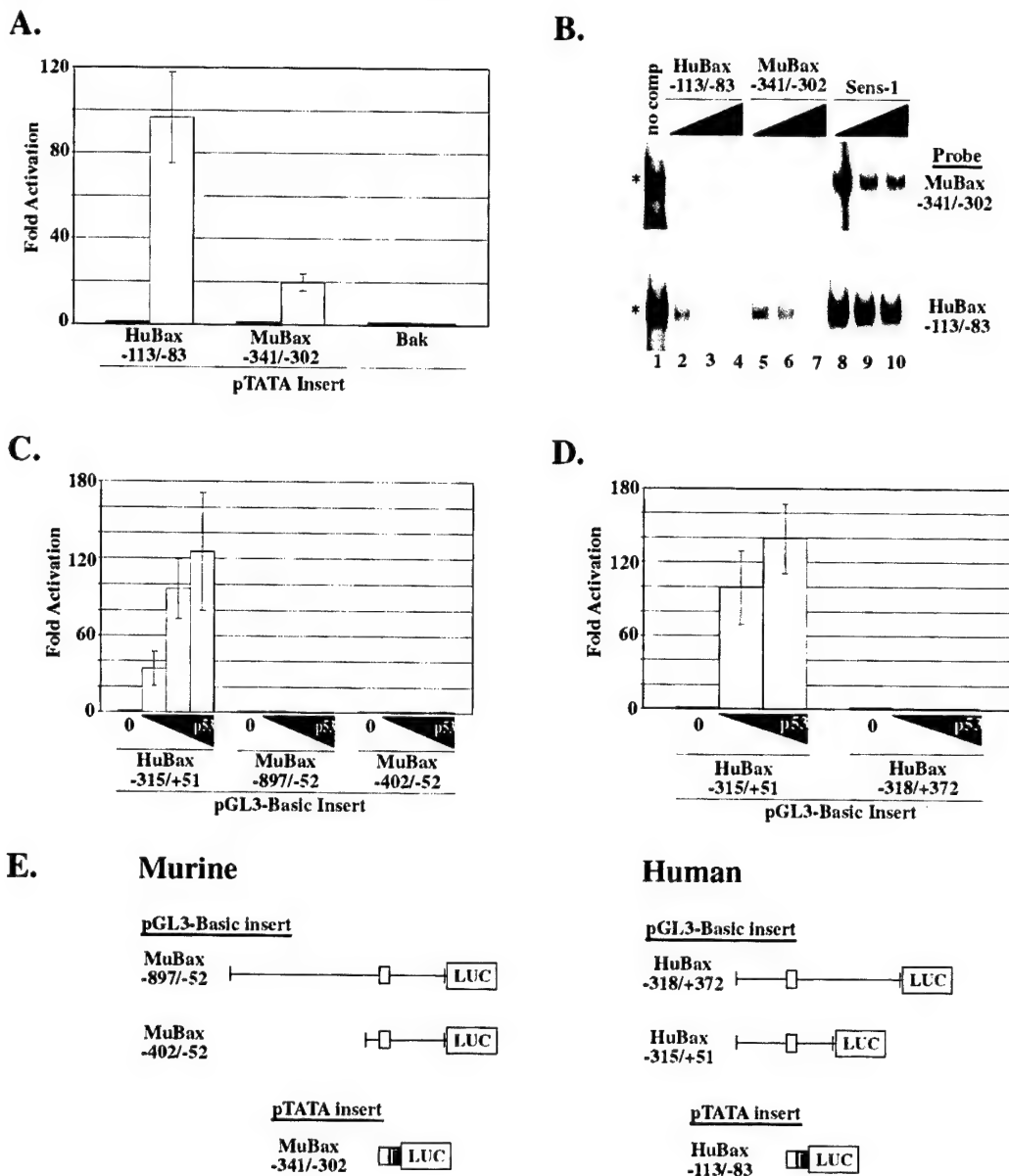
To determine if there is functional conservation of the promoter response element, synthetic oligonucleotides corresponding to -113/-83 in the human *bax*



**Figure 1** Cloning of the murine *bax* gene demonstrates sequence conservation of the promoter p53 response element. The murine *bax* gene from -898 to +542 from the start site of translation was PCR cloned (Accession # AF339055) from mouse genomic DNA. This region is schematically represented with the black boxes indicating the positions of exons 1 (+1 to +34) and 2 (partial sequence: +505 to +542). The white box labeled -339 to -304 indicates a region of conservation between the mouse and human sequences. The nucleotide sequences of this region are listed. For comparison the region of the human *bax* gene that demonstrates conservation is listed above the mouse sequence. The numbering of the human sequence is with respect to the start site of transcription. A solid line between the two sequences indicates perfect conservation at that base. Dashed lines indicate a conservative substitutions of purine for purine or pyrimidine for pyrimidine. The white boxes, labeled 1-3, indicate the p53 response element half-sites from the human gene and the corresponding murine sequences. The asterisks indicate bases that deviate from the p53 DNA-binding consensus sequence. The arrow indicates a cytosine at position -332 that was not reported in the original cloning of the murine *bax* promoter (Igata et al., 1999)

gene and to -341/-302 in the murine *bax* gene were cloned upstream of a minimal promoter and cotransfected into the p53-deficient Saos-2 cell line with an expression vector for wild-type p53 (Figure 2a). Wild-type p53 effectively activated transcription through the response element from the human *bax* promoter (Figure 2a, HuBax-113/-83). The murine *bax* sequence also was activated significantly by p53 (Figure 2a, MuBax-342/-302). The level of activation observed with the murine element, however, was reduced as compared to that seen with the human sequence (Figure 2a, compare 98-fold with HuBax-113/-83 to 19-fold with MuBax-341/-302). A reporter containing an unrelated sequence from the human *bak* promoter did not respond to p53 (Figure 2a, Bak). Similar results were observed in cotransfection assays using an expression vector for wild-type murine p53 (data not shown).

The ability of p53 to directly bind the murine sequence from -341/-302 was addressed using electrophoretic mobility shift assay (EMSA). Oligonucleotides corresponding to either this sequence (Figure 2b, upper panel) or the human sequence from -113/-83 (Figure 2b, lower panel) were used as radiolabeled probes in EMSAs. Purified p53 bound both radiolabeled probes (Figure 2b, lane 1), and was effectively competed by increasing amounts of unlabeled human and murine *bax* oligonucleotides (Figure 2b, lanes 2-4 and 5-7 respectively). An unrelated oligonucleotide, Sens-1, failed to compete for p53 binding with either

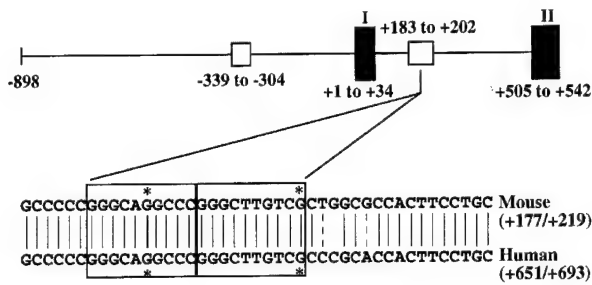


**Figure 2** The p53 response element from the promoter of the human *bax* gene is functionally conserved in the mouse. (a) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pTATA reporter plasmids in the presence of either 50 ng of pCMV (black bars) or 50 ng of pCMV-p53<sup>wt</sup> (white bars). Twenty-four hours post transfection cells were lysed and assayed for total protein and luciferase activity. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. (b) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to either the -113/-83 sequence from the human *bax* promoter (bottom panel) or the -341/-302 sequence from the murine *bax* gene (top panel) as radiolabeled probe. Fifty ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled HuBax -113/-83 oligonucleotide (lanes 2-4), the MuBax -341/-302 oligonucleotide (lanes 5-7), or the Sens-1 oligonucleotide (lanes 8-10). The asterisks indicate the positions of the p53-DNA complexes. (c) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pGL3 reporter plasmids in the presence of 0, 25, 50, or 100 ng of pCMV-p53<sup>wt</sup>. (d) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pGL3 reporter plasmids in the presence of 0, 25, 50, or 100 ng of pCMV-p53<sup>wt</sup>. (e) Where appropriate, amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of total plasmid DNA. Twenty-four hours post transfection cells were lysed and assayed for total protein and luciferase activity. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. (e) Luciferase reporter constructs used in (a), (c), and (d) are diagrammed with the smaller white boxes representing the p53 response element, the gray boxes representing the adenovirus *E1b* minimal promoter, and the larger white boxes labeled 'LUC' representing the luciferase coding domain.

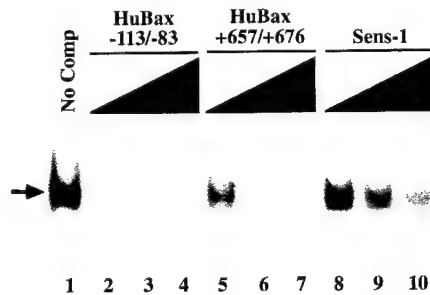
probe (Figure 2b, lanes 8-10), demonstrating that the binding of p53 to both the human and murine *bax* promoter elements is specific.

Reporter plasmids were constructed containing either the -897/-52 or the -402/-52 sequence from the start site of translation of the murine *bax* promoter

A.



B.



**Figure 3** Cloning of the *bax* gene reveals a potential p53 response element in the first intron that is conserved between humans and mice. (a) The murine *bax* gene from -898 to +542 from the start site of translation is schematically represented with the black boxes indicating the positions of exons 1 (+1 to +34) and 2 (partial sequence: +505 to +542). The white box labeled -339 to -304 indicates the previously described p53 response element (see Figure 1). The white box labeled +183 to +202 indicates a region of conservation between the mouse and human sequences. The nucleotide sequences of this region is listed. For comparison the region of the human *bax* gene (Accession #AF339054) that demonstrates conservation is listed below the mouse sequence. The numbering of the human sequence is with respect to the start site of transcription. A solid line between the two sequences indicates perfect conservation at that base. Dashed lines indicate conservative substitutions of purine for purine or pyrimidine for pyrimidine. The asterisks indicate bases that deviate from the p53 DNA-binding consensus sequence. (b) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the +657/+676 sequence from the human *bax* gene (identical to +183/+202 in the mouse) as radiolabeled probe. Fifty ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled HuBax-113/-83 oligonucleotide (lanes 2-4), the HuBax+657/+676 oligonucleotide (lanes 5-7), or the Sens-1 oligonucleotide (lanes 8-10). The arrow indicates the position of the p53-DNA complex

to test the significance of the response element at -339/-304 in the context of the *bax* promoter. Consistent with previously published results (Igata *et al.*, 1999; Schmidt *et al.*, 1999), p53 failed to mediate transcriptional activation through either murine promoter construct while successfully activating a reporter plasmid containing -315/+51 from the start site of transcription of the human *bax* promoter (Figure 2c). These data suggest a discrepancy between the ability of p53 to activate transcription through the human *bax* promoter as compared to the murine promoter. To examine this further, the ability of a larger fragment of

the human promoter to mediate p53-dependent activation was tested. p53 failed to activate a reporter plasmid containing -318/+372 from the start site of transcription of the human *bax* promoter while successfully activating the smaller -315/+51 promoter construct (Figure 2d), demonstrating that, as seen with the mouse promoter, the p53 binding site in the context of a larger fragment of the human *bax* promoter is also unresponsive to p53. These results suggest that a negative regulatory element may exist between +51 and +372 in the 5' untranslated region (5'UTR) of the human *bax* gene that inhibits the ability of p53 to mediate activation through its binding site in the promoter.

#### Cloning of the human and murine *bax* genes identifies a novel p53 response element in the first intron that is required for p53-dependent activation

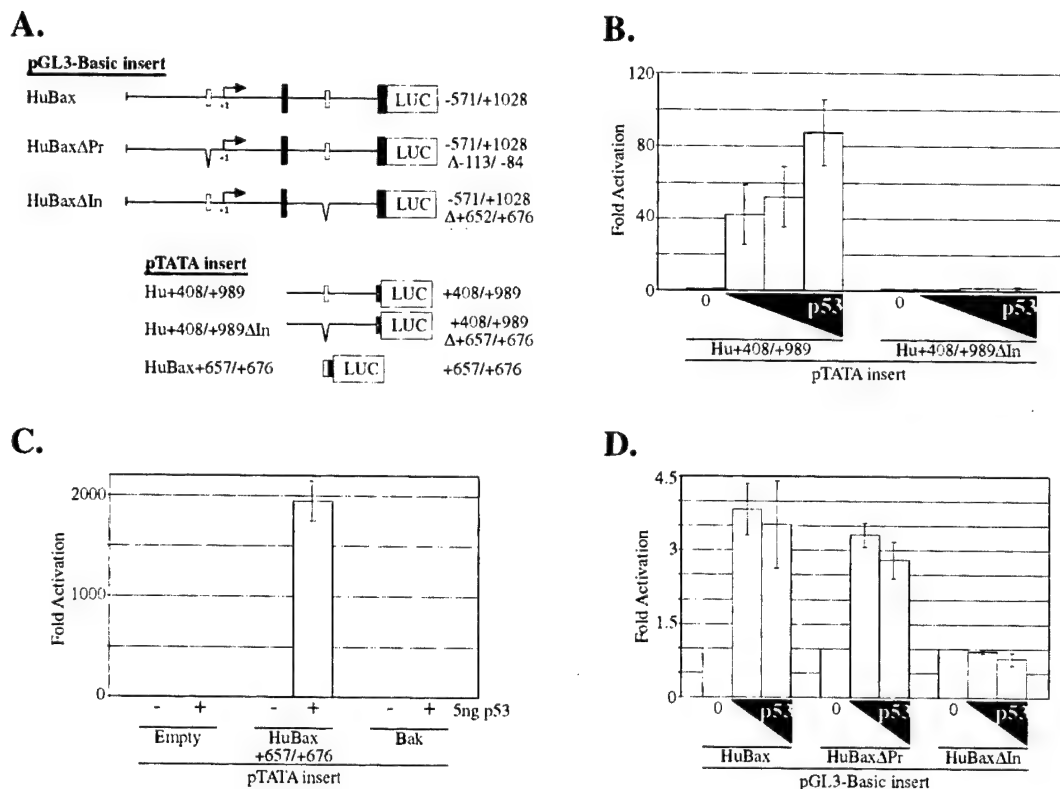
Since the *bax* promoter was unresponsive to p53, the murine *bax* gene was further analysed for additional sequences that may participate in the p53-dependent regulation of *bax* transcription. Sequence analysis of the first intron revealed 20 bp at +183/+202 from the start site of translation that match the p53 DNA-binding consensus sequence at 18 of 20 bases (Figure 3a, white boxes, mismatches indicated by asterisks). This sequence was confirmed in five independent PCRs of mouse genomic DNA. To assess whether or not this sequence is evolutionarily conserved, a 1599 bp fragment (-571/+1028 from the start site of transcription) of the *bax* gene was PCR cloned from human genomic DNA (Accession #AF339054). A 43 bp region at +651/+693 was found to be 93% identical to the murine *bax* sequence at +177/+219 (Figure 3, solid lines represent identities, dashed lines represent conservative substitutions). Further, the putative p53 response element at +183/+202 in the mouse gene is perfectly conserved in the first intron of the human gene at +657/+676 from the start site of transcription. The human sequence was confirmed in three independent PCRs of human genomic DNA, and, subsequently, has been confirmed independently by the Human Genome Project (Accession #11425312).

To determine whether the sequence from +657/+676 in the human *bax* gene (+177/+219 in the murine *bax* gene) constitutes a *bona fide* p53 response element, a synthetic oligonucleotide corresponding to this sequence was used as a radiolabeled probe in an EMSA with purified p53 (Figure 3b). Purified p53 bound the labeled oligonucleotide containing the putative response element (Figure 1b, lane 1), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Figure 3b, lanes 5-7). An oligonucleotide corresponding to the p53 response element at -113/-83 from the start of transcription in the human promoter also competed for p53 binding (Figure 3b, lanes 2-4). An unrelated control oligonucleotide, Sens-1, was unable to compete for p53 binding (Figure 3b, lanes 8-10), demonstrating that the binding of p53 to +657/+676 is sequence-specific.

The significance of this 20 bp element to p53-dependent activation in cells was addressed in transient transfection assays. Luciferase reporter plasmids containing different regions of the human *bax* gene were constructed (Figure 4a) and cotransfected with a wild-type p53 expression vector into the Saos-2 cell line (Figure 4). When cloned upstream of a minimal promoter, the first intron of the human *bax* gene (Figure 4a, Hu+408/+989) mediated a significant degree of activation in response to p53 (Figure 4b, Hu+408/+989). Deletion of the putative p53 response element at +657/+676 (Figure 4a, Hu+408/+989 $\Delta$ In) abolished the ability of p53 to transcriptionally activate (Figure 4b, Hu+408/+989 $\Delta$ In), demonstrating that the first intron of the human *bax* gene can confer p53 responsiveness upon a minimal promoter and that the p53 response element at +657/+676 is required for this activity. To determine if this 20 bp element is sufficient to mediate p53-dependent transcriptional activation, an oligonucleotide corre-

sponding to this sequence was cloned into the minimal promoter reporter (Figure 4a, HuBax+657/+676). This reporter plasmid mediated a significant degree of activation in response to p53 (Figure 4c, HuBax+657/+676). Thus, the 20 bp from +657/+676 is sufficient to confer p53-dependent transcriptional activation upon a minimal promoter. A reporter with no insert as well as a reporter containing an unrelated sequence from the human *bak* promoter failed to respond to wild-type p53 (Figure 4c, Empty and Bak). Similar results were also obtained with a reporter containing a p53 response element from the human p21 promoter which had been mutated such that it no longer bound to p53 as determined by EMSA (data not shown).

To address the significance of this intronic p53 response element in the larger context of the human *bax* gene, a reporter plasmid was made in which the *bax* gene from -571 to +1028 was cloned in frame with luciferase, forming a fusion between exon 2 of *bax* and the luciferase of pGL3-Basic (Figure 4a, HuBax).



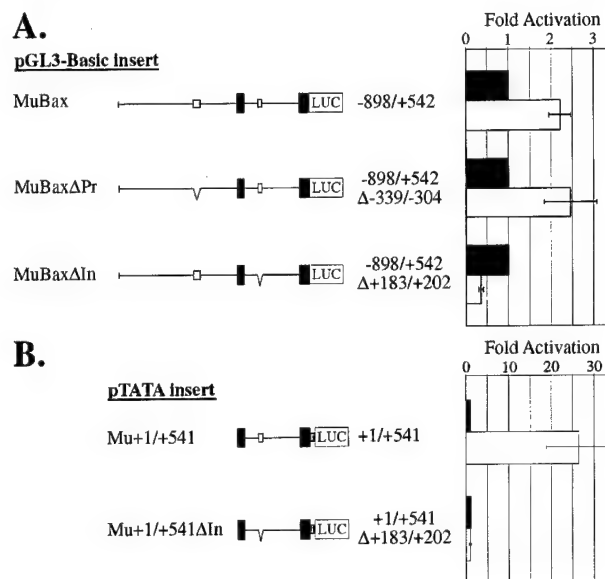
**Figure 4** The first intron of the human *bax* gene contains a p53 response element that is required for p53-dependent transcriptional activation. (a) Schematic representation of the HuBax, HuBax $\Delta$ Pr, HuBax $\Delta$ In, Hu+408/+989, Hu+408/+989 $\Delta$ In, and HuBax+657/+676 reporter plasmids. Exons are represented by black boxes, and p53 response elements are represented by white boxes. The gray boxes represent the adenovirus *Elb* minimal promoter. The arrow labeled +1 indicates the start site of transcription. Deletions are indicated by the downward pointing notches. The nucleotides contained within each plasmid are indicated to the right. Numbers are in reference to the start site of transcription. (b) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pTATA reporter plasmids in the presence of 0, 1, 5, and 10 ng of pCMV-p53<sup>wt</sup>. (c) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pGL3 reporter plasmids in the presence of either 5 ng of pCMV (-) or 5 ng of pCMV-p53<sup>wt</sup> (+). (d) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pGL3 reporter plasmids in the presence of 0, 100, and 200 ng of pCMV-p53<sup>wt</sup>. Where appropriate, amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of total plasmid DNA. Twenty-four hours post transfection cells were lysed and assayed for total protein and luciferase activity. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation.



When transfected into Saos-2 cells, this reporter plasmid mediated a significant degree of transcriptional activity in response to a cotransfected p53 expression vector (Figure 4d, HuBax). There was no substantial difference between the p53 responsiveness of this reporter as compared to a reporter in which the previously characterized p53 response element at -113/-83 is deleted (Figure 4a, HuBax $\Delta$ Pr) (Figure 4d, compare 3.5–3.8-fold with HuBax to 2.8–3.3-fold with HuBax $\Delta$ Pr). Deletion of the intronic region containing the newly identified p53 response element (Figure 4a, HuBax $\Delta$ In), however, produced a reporter that was unresponsive to wild-type p53 (Figure 4d, HuBax $\Delta$ In), demonstrating that this element is required for p53 to mediate transcriptional activation of the human *bax* gene.

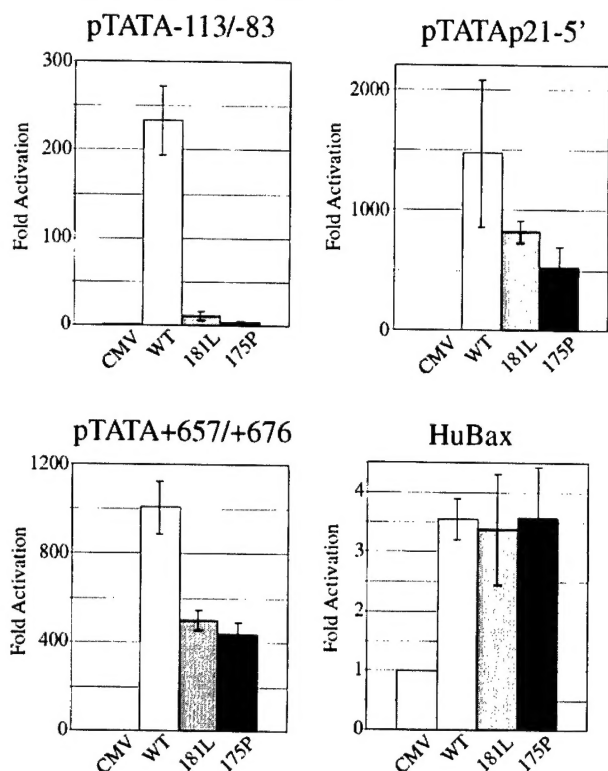
Given the perfect conservation of the intronic p53 binding site, the above results with the human *bax* reporter plasmids (Figure 4b,d) were compared with a similar set of constructs based on the murine gene. A reporter plasmid was made in which the murine *bax* gene from -898 to +542 from the start site of translation was cloned in frame with luciferase, again forming a fusion between exon 2 of *bax* and the luciferase of pGL3-Basic (Figure 5a, MuBax). When cotransfected with a p53 expression vector into Saos-2 cells this reporter plasmid was transcriptionally activated (Figure 5a, MuBax). As observed with the human *bax* gene (Figure 4d), there was no significant difference between the p53-dependent transactivation of this reporter as compared to a reporter in which the p53 response element at -339/-304 is deleted (Figure 5a, compare MuBax to MuBax $\Delta$ Pr). Deletion of the intronic region containing the newly identified p53 response element, however, produced a reporter construct that was unresponsive to wild-type p53 (Figure 5a, MuBax $\Delta$ In). Also, the isolated intron from the murine *bax* gene conferred p53-responsiveness on a minimal promoter, while this same reporter with the p53 response element at +183/+202 deleted did not (Figure 5b). Together, these results demonstrate that this intronic element is required for p53 to mediate transcriptional activation of the murine *bax* gene, as was the case with the human gene.

Two tumor-derived mutants of p53, 181L and 175P, have been shown to be capable of activating transcription through the p21 promoter but to fail to do so through the *bax* promoter. This failure to activate the *bax* promoter correlated with the inability of these mutants to induce apoptosis (Ludwig et al., 1996). The ability of the 181L and 175P p53 mutants to activate transcription through various p21 and *bax* reporter plasmids was assayed by transient transfection in Saos-2 cells (Figure 6). Wild-type p53 (Figure 6, white bars) successfully activated transcription through each reporter construct assayed. In addition to wild-type p53, the 181L (dark gray bars) and 175P (black bars) mutants also mediated a significant amount of activation through the 5' p53 response element of the human p21 promoter (p21-5') cloned upstream of a minimal promoter (Figure 6, pTATAp21-5'). Consis-



**Figure 5** The first intron of the murine *bax* gene contains a p53 response element that is required for p53-dependent transcriptional activation. (a) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pGL3 reporter plasmids in the presence of either 100 ng of pCMV (black bars) or 100 ng of pCMV-p53<sup>wt</sup> (white bars). (b) Saos-2 cells were transfected with 1  $\mu$ g of the indicated pTATA reporter plasmids in the presence of either 10 ng of pCMV (black bars) or 10 ng of pCMV-p53<sup>wt</sup> (white bars). (a) and (b) Twenty-four hours post transfection cells were lysed and assayed for total protein and luciferase activity. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. Each reporter is diagrammed with black boxes representing exons, white boxes indicating p53 response elements, and gray boxes representing the adenovirus *Elb* minimal promoter. Deletions are indicated by downward pointing notches. The nucleotides contained within each construct are indicated to the right.

tent with studies using the *bax* promoter, however, these mutants failed to activate transcription through the p53 response element of the human *bax* promoter (Figure 6, pTATA-113/-83). In contrast to -113/-83, yet similar to p21-5', the 181L and 175P mutants successfully activated transcription through the intronic p53 response element of the *bax* gene (Figure 6, pTATA+657/+676) as well as through the HuBax reporter (Figure 6, HuBax), which contains -571/+1028 of the human *bax* gene cloned in frame with luciferase (see Figure 4a). In the case of both the *bax* intronic and p21-5' elements the levels of activation observed with the p53 mutants were less than that observed with wild-type p53. This is consistent with the observation that, as compared to wild-type p53, the two mutants were expressed at a slightly lower level in Saos-2 cells (data not shown). The effect of these mutant proteins on endogenous *bax* expression was then examined. Saos-2 cells were transfected with expression plasmids for wild-type p53 as well as the 175P and 181L mutant p53 proteins. Levels of endogenous p21, *bax*, and GAPDH mRNA were then determined by RT-PCR (Figure 7). Expression of all three p53 proteins caused up-regulation of both p21

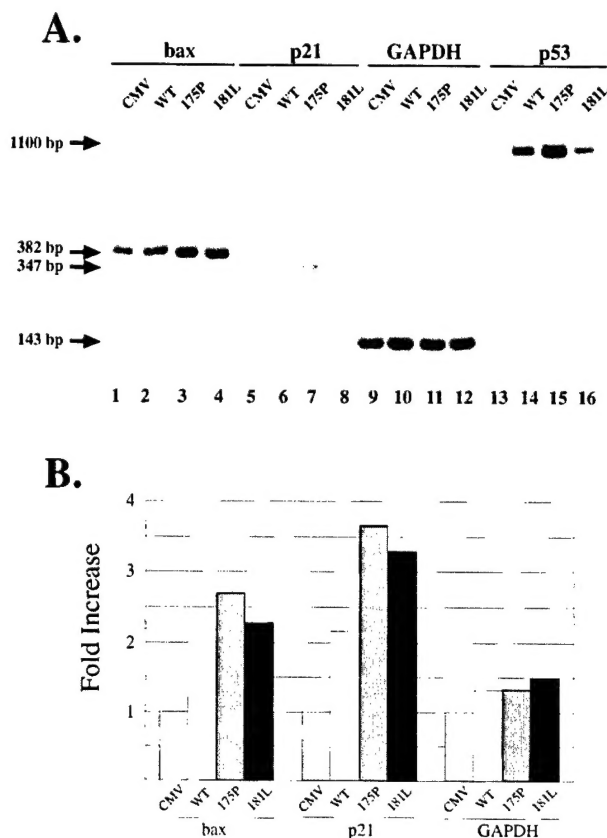


**Figure 6** The p53 mutants 181L and 175P successfully activate transcription through the intronic response element. Saos-2 cells were transfected with 1  $\mu$ g of the indicated reporter plasmids in the presence of either 100 ng (HuBax) or 10 ng (pTATA-113/-83, +657/+676, and p21-5') of pCMV (light gray bars), pCMV-p53<sup>WT</sup> (white bars), 181L (dark gray bars), or 175P (black bars). Twenty-four hours post transfection cells were lysed and assayed for total protein and luciferase activity. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation

and *bax* mRNA without affecting the level of GAPDH mRNA. Since these mutants are transcriptionally inactive on the p53 site in the *bax* promoter, this ability of the mutant p53 proteins to increase endogenous *bax* mRNA levels supports a role for the intronic element in p53-dependent up-regulation of endogenous *bax* expression.

## Discussion

Thymocytes from the *bax* knock-out mouse continue to undergo p53-dependent apoptosis in response to ionizing radiation, suggesting that Bax up-regulation is not necessary (Knudson et al., 1995). Recent studies with the *bax/bak* double knock-out mouse have shown that *bax* does indeed play an important role in this process. These experiments demonstrated that while thymocytes from either the *bax* or the *bak* knock-out mouse undergo p53-dependent apoptosis, thymocytes from the *bax/bak* double knock-out do not, suggesting that these two gene products are functionally redundant in this cell type and are together important



**Figure 7** The p53 mutants 181L and 175P up-regulate expression of endogenous *bax* mRNA. (a) Saos-2 cells were transfected with 5  $\mu$ g of the indicated expression plasmids: pCMV, pCMV-p53<sup>WT</sup>, 181L, or 175P. Twenty-four hours post transfection RNA was isolated and subjected to RT-PCR analysis using primers for *bax*, *p21*, *GAPDH*, and *p53*. (b) RT-PCR analysis was quantitated by phosphorimaging. The indicated values are the average of three independent experiments

downstream effectors of p53-dependent apoptosis (Lindsten et al., 2000). Wei et al. (2001) have further substantiated these results in cell culture showing that either *bax* or *bak* is required for apoptosis triggered by almost all forms of stimuli, including those that are p53-dependent. In fact, p53-dependent regulation of Bax expression has been observed in the prostate, thymus, spleen, small intestine, and lung, as well as sympathetic, Purkinje, and olfactory cortical neurons of the mouse (Miyashita et al., 1994; Bouvard et al., 2000). Further, Bax has been shown both to be an obligatory downstream effector for the p53-mediated apoptosis that attenuates choroid plexus tumor growth in the TgT121 mouse model (Yin et al., 1997), and to function as an effector of p53-dependent apoptosis and a suppressor of oncogenic transformation in mouse embryo fibroblasts (McCurach et al., 1997). Studies showing that the mouse *bax* promoter is not p53 responsive, however, raised the question of whether *bax* is a direct transcriptional target of p53 in the mouse (Igata et al., 1999; Schmidt et al., 1999). In humans, Zhang et al. (2000) demonstrated in colorectal cancer cells that *bax* is responsible for approximately



50% of the p53-dependent apoptosis observed in response to 5-fluorouracil treatment. While *Bax* is clearly not the only downstream effector in p53-dependent apoptosis, it does contribute significantly to this response. Although Miyashita and Reed (1995) identified a p53 response element in the human *bax* promoter, a recent study, subsequently confirmed here (Figure 2d), showed that a reporter containing 1 kb of promoter sequence fails to respond to p53 (Igata *et al.*, 1999).

The data presented in this report demonstrate the existence of a novel p53 response element in the first intron of the *bax* gene (Figures 3–5). This element is found to be perfectly conserved between the human and murine *bax* genes (Figure 3). Further, it is shown that this element is both sufficient and necessary for p53 to mediate transcriptional activation of the *bax* gene in both species (Figures 4 and 5). In addition, we have demonstrated partial conservation of the p53 response element of the human *bax* promoter in the murine promoter at –339 to –304 (Figures 1 and 2). This sequence from the murine *bax* promoter is capable of binding p53 in a sequence specific manner *in vivo* and of conferring p53-dependent transcriptional activation upon a minimal promoter in cells (Figures 2a,b). Consistent with previously published data (Igata *et al.*, 1999), however, the promoter element, in the context of both the human and murine *bax* genes, appears to be neither sufficient nor necessary for p53-dependent transactivation (Figures 2, 4 and 5), at least in the cell line and under the conditions examined here. The tumor-derived p53 mutants, 181L and 175P, which are defective in inducing an apoptotic response (Ludwig *et al.*, 1996) retain the ability to activate transcription via the *bax* intronic p53 site. Since these mutants are transcriptionally inactive on the p53 site in the *bax* promoter, the ability of these mutants to up-regulating endogenous *bax* mRNA levels supports a role for the intronic element in p53-dependent up-regulation of *bax* expression. Further, the results with the human *bax* promoter demonstrate that the addition of 321 bp of the 5'UTR renders an otherwise responsive *bax* promoter unresponsive to p53 (Figure 2d). This result suggests that an inhibitory sequence exists in the 5'UTR of *bax*, and offers the possibility that the promoter element may be functional in cells in which this inhibition does not occur.

Clearly the p53-dependent regulation of *bax* is complex likely involving two unique p53 response elements, one in the promoter and one in the first intron, as well as a potential negative regulatory element in the 5'UTR. The results presented here, however, do demonstrate that both the human and murine *bax* genes can function as direct transcriptional targets of the tumor suppressor protein p53, and that this response appears to involve a newly identified response element in the first intron. Thus, these results confirm and substantiate the important role of *bax* as a direct and evolutionarily conserved target of p53-dependent transcriptional activation.

## Materials and methods

### Cells

The osteosarcoma Saos-2 cell line was maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells were grown in Dulbecco's modified Eagle's medium, containing 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

### Oligonucleotides

For use in electrophoretic mobility shift assays and for subsequent cloning into luciferase reporter plasmids, complementary single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides with the indicated sequences: HuBax–113/–83, AATTCGGTACCTCACAA-GTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAA-TT; HuBax+657/+676, AATTCGGTACCGGGCAGGCC-CGGGCTTGTCGGCTAGCGAATT; MuBax–341/–302, AATTCGGTACCGATGACAAGCATATCCCAGGCAAG-CTTTGAACCTTGCGGCAGCTAGCGAATT; MuBax–341/–315, AATTCGGTACCGATGACAAGCATATCCCAGG-CAAGCTTGCTAGCGAATT; MuBax–324/–315, AATTCGGTACCGGCAAGCTTTGAACCTTGCGGCAGCTA-GCGAATT; Bak, AATTCGCTAGCGGGCCAGGCCAG-GAGCATGTCCCCGCCCACTTTCCCGGTACCGAATT; Sens-1, AATTCGGTACCTCGAAGAAGACGTGCAAG-GACCCGCTAGCGAATT; p21-5', AATTCGGTACCGAA-CATGTCCCAACATGTTGGCTAGCGAATT.

### Plasmids

With respect to the human *bax* gene, numbering is in reference to the start of transcription, while in the mouse gene numbering is from the start of translation. The following synthetic double-stranded oligonucleotides were cloned into the *Kpn*I and *Nhe*I restriction sites of pTATA, originally referred to as pGL3-E1bTATA (Resnick-Silverman *et al.*, 1998), to produce vectors with corresponding names: HuBax–113/–83, HuBax+657/+676, MuBax–341/–302, MuBax–341/–315, MuBax–324/–315, p21-5', and Bak. Hu+408/+989 was generated by PCR amplification of intron 1 (+408 to +989) of the *bax* gene from human genomic DNA (Clontech). PCR primers contained appropriate sequence for the subsequent cloning of this product into the *Eco*RI and *Sac*I sites of the pTATA reporter plasmid. A PCR-based strategy was used to replace the region in Hu+408/+989 corresponding to +657 to +676 with the *Nco*I restriction site to produce Hu+408/+989ΔIn. Mu+1/+541 was generated by PCR amplification of +1 to +541 of the *bax* gene from murine genomic DNA (Clontech), and subsequent cloning of this product into the *Kpn*I and *Nhe*I sites of the pTATA reporter plasmid. The region of Mu+1/+541 corresponding to +183 to +202 was replaced with the *Sac*I restriction site to produce Mu+1/+541ΔIn.

HuBax was generated by PCR amplification of –571 to +1028 of the *bax* gene from human genomic DNA (Clontech). PCR primers were based on previously published promoter and exon sequence data and contained the appropriate sequence for the subsequent cloning of this product into the *Mlu*I and *Nco*I restriction sites of the pGL3-Basic reporter plasmid, maintaining the reading frame between the *bax* coding domain and the *luciferase* of pGL3-Basic. HuBaxΔPr and HuBaxΔIn were derived from HuBax by replacing the relevant sequences, –113 to –84

and +652 to +676 respectively, with the *Bgl*II restriction site. HuBax-315/+51, originally referred to as pBax-315/+51, was as described previously (Thornborrow and Manfredi, 2001). HuBax-318/+372 was generated by PCR amplification of -318 to +372 of the *bax* gene from HuBax. The PCR product was cloned into the *Nhe*I and *Hind*III restriction sites of pTATA, replacing the adenovirus *E1b* minimal promoter.

MuBax was generated by PCR amplification of -898 to +542 of the *bax* gene from mouse genomic DNA (Clontech). Primers were based on previously published promoter and exon sequence. This PCR product was cloned into the *Kpn*I and *Nco*I restriction sites of the pGL3-Basic reporter plasmid, maintaining the reading frame between the *bax* coding domain and the *luciferase* of pGL3-Basic. MuBax $\Delta$ Pr and MuBax $\Delta$ In were derived from MuBax by replacing the relevant sequences, -339 to -304 and +183 to +202 respectively, with the *Bgl*II restriction site. MuBax-897/-52 and MuBax-402/-52 were generated by PCR amplification from mouse genomic DNA (Clontech). These PCR products were cloned into the *Kpn*I and *Nhe*I restriction sites of the pGL3-Basic reporter plasmid. The validity of all reporter plasmids was confirmed by sequencing.

The expression plasmid pCMV-p53<sup>wt</sup>, originally referred to as pC53-SN3 (Baker et al., 1990), encodes the wild-type human p53 protein under the control of the cytomegalovirus promoter. The mutant p53 expression vectors 181L and 175P encode the corresponding p53 mutant under the control of the cytomegalovirus promoter (Ludwig et al., 1996).

#### Transfections

SaOs-2 cells were transfected using Lipofectamine Plus Reagent (GibcoBRL, Life Technologies).  $2 \times 10^5$  cells were seeded into 35-mm plates. Cells were transfected 24 h later according to the manufacturer's instructions. Cellular lysates were prepared 24 h post-transfection, total protein concentration was determined by protein assay (Bio-Rad), and luciferase assays were quantitated using a commercially available kit (Promega) and a TD-20c Luminometer (Turner).

#### Electrophoretic mobility shift assay

Production of baculovirus-infected Sf9 cell extracts and purification of recombinant human p53 protein was done as previously described (Resnick-Silverman et al., 1998). Purified p53 protein was incubated with 3 ng of radiolabeled double-stranded oligonucleotide in a total volume of 30  $\mu$ l of DNA-binding buffer (20 mM HEPES pH 7.5, 83 mM NaCl, 0.1 mM EDTA, 12% glycerol, 2 mM MgCl<sub>2</sub>, 2 mM spermidine, 0.7 mM DTT, and 25  $\mu$ g/ml poly d(I-C)) for 20 min at room temperature. Samples were loaded on a native 4%

acrylamide gel in 0.5 $\times$  TBE and electrophoresed at 4°C at 225 V for 2 h. The gel was dried and exposed to Kodak XAR film using an intensifying screen at -70°C.

#### RT-PCR

SaOs-2 cells were transfected using Lipofectamine Plus Reagent (GibcoBRL, Life Technologies).  $1 \times 10^6$  cells were seeded into 100-mm plates. Cells were transfected 24 h later according to the manufacturer's instructions. RNA was isolated with Ultraspec (Biotecx) 24 h post-transfection, and 1  $\mu$ g was used immediately for cDNA synthesis with SuperScript RT (Life Technologies) according to the manufacturer's instructions. Primers used for PCR amplification were: *bax*, 5'-CCCTGGACCCGGTGCCTCAGG-3'/5'-TCACGGTCTGCCACGTGGGCGT-3'; *p21*, 5'-GGGGGCATCATCAAAACTT-3'/5'-ACTGAAGGGAAAGGACAAGG-3'; *GAPDH*, 5'-GTGAAGGTCGGAGTCAAC-3'/5'-TGGAA-TTGCCATGGGTG-3'; *p53*, 5'-CGCGGATCCATGGAGGAGCCGCAGTCAG-3'/5'-CCCAAGCTTGTCACTCTGAGTCAGGCC. PCR reactions were set up containing 1 $\times$  PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 150 ng of each primer, 2.5 units PLATINUM Taq DNA Polymerase (Life Technologies), 5% DMS, 2  $\mu$ l template cDNA, 0.25  $\mu$ Ci  $\alpha$ -<sup>32</sup>P dATP and distilled water to 50  $\mu$ l. PCR conditions for *p21*, *GAPDH* and *p53* were 25 cycles: 45 s at 94°C, 45 s at 55°C and 1 min at 72°C. *Bax* PCR was as above except the annealing temperature was at 65°C. Samples were run on a 6% acrylamide gel and visualized using autoradiography. Phosphorimaging was performed with a Personal Molecular Imager FX and analysed with Quantity One software (Bio-Rad).

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